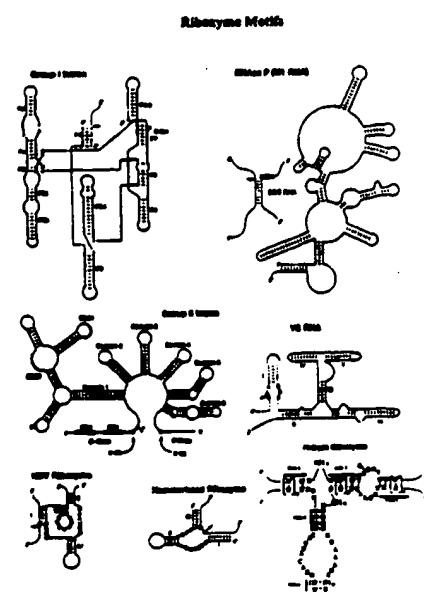




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: <b>PCT/US98/09249</b></p> <p>(22) International Filing Date: 5 May 1998 (05.05.98)</p> <p>(30) Priority Data:</p> <table> <tr><td>60/046,059</td><td>9 May 1997 (09.05.97)</td><td>US</td></tr> <tr><td>60/049,002</td><td>9 June 1997 (09.06.97)</td><td>US</td></tr> <tr><td>60/051,718</td><td>3 July 1997 (03.07.97)</td><td>US</td></tr> <tr><td>60/056,808</td><td>22 August 1997 (22.08.97)</td><td>US</td></tr> <tr><td>60/061,324</td><td>2 October 1997 (02.10.97)</td><td>US</td></tr> <tr><td>60/061,321</td><td>2 October 1997 (02.10.97)</td><td>US</td></tr> <tr><td>60/064,866</td><td>5 November 1997 (05.11.97)</td><td>US</td></tr> <tr><td>60/068,212</td><td>19 December 1997 (19.12.97)</td><td>US</td></tr> </table> <p>(71) Applicant (for all designated States except US): <b>RIBOZYME PHARMACEUTICALS, INC. [US/US]</b>; 2950 Wilderness Place, Boulder, CO 80301 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): <b>JARVIS, Thale [US/US]</b>; 3720 Smuggler Place, Boulder, CO 80301 (US). <b>MATULIC-ADAMIC, Jasenka [YU/YU]</b>; 760 South 42nd Street, Boulder, CO 80303 (US). <b>REYNOLDS, Mark [US/US]</b>; 4184 N. Larkspur Court, Lafayette, CO 80026 (US). <b>KISICH, Kevin [US/US]</b>; 2451 Tonquil Circle, Lafayette, CO 80026 (US). <b>BELLON, Laurent [FR/FR]</b>.</p>		60/046,059	9 May 1997 (09.05.97)	US	60/049,002	9 June 1997 (09.06.97)	US	60/051,718	3 July 1997 (03.07.97)	US	60/056,808	22 August 1997 (22.08.97)	US	60/061,324	2 October 1997 (02.10.97)	US	60/061,321	2 October 1997 (02.10.97)	US	60/064,866	5 November 1997 (05.11.97)	US	60/068,212	19 December 1997 (19.12.97)	US	<p>2946 Glenwood Drive, Boulder, CO 80301 (US). <b>PARRY, Tom [US/US]</b>; 12610 Utica Street, Broomfield, CO 80020 (US). <b>BEIGELMAN, Leonid [US/US]</b>; 5530 Colt Drive, Longmont, CO 80503 (US). <b>MCSWIGGEN, James, A. [US/US]</b>; 4866 Franklin Drive, Boulder, CO 80301 (US). <b>KARPEISKY, Alexander [US/US]</b>; 420 Vernier Avenue, Lafayette, CO 80024 (US). <b>BURGIN, Alex [US/US]</b>; 832 Caminito Estrella, Chula Vista, CA 91910 (US). <b>THOMPSON, James [US/US]</b>; 2925 Glenwood Drive #103, Boulder, CO 80301 (US). <b>WORKMAN, Christopher, T. [US/US]</b>; 3910 Longwood, Boulder, CO 80303 (US). <b>BEAUDRY, Amber [US/US]</b>; 711 E. Genesee Street, Lafayette, CO 80026 (US). <b>SWEEDLER, David [US/US]</b>; 956 St. Andrews Lane, Louisville, CO 80027 (US).</p> <p>(74) Agents: <b>WARBURG, Richard, J. et al.</b>; Lyon &amp; Lyon, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).</p> <p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i></p>	
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<p>(54) Title: <b>ENZYMATIC NUCLEIC ACID TREATMENT OF DISEASES OR CONDITIONS RELATED TO LEVELS OF EXPRESSION OF C-RAF</b></p> <p>(57) Abstract</p> <p>Nucleic acid catalysts which modulate the expression of Raf gene; method of delivery, screening, identification, synthesis, deprotection, purification, of nucleic acid catalysts and processes for identification of nucleic acid molecules is described.</p>																											
																											

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DESCRIPTIONENZYMATIC NUCLEIC ACID TREATMENT OF DISEASES OR CONDITIONS  
RELATED TO LEVELS OF EXPRESSION OF C-RAFBackground Of The Invention

5 This invention relates to methods and reagents for the treatment of diseases or conditions relating to the levels of expression of *raf* genes.

The following is a discussion of relevant art, none of which is admitted to be prior art to the present invention.

10 The Raf family of serine/threonine kinases function as cytoplasmic signaling proteins that transduce mitogenic signals in response to activation of various growth factor receptors (for reviews, see Daum, 1994 *Trends in Biochem. Sci.* 19, 474; Katz, 1997, *Curr. Opin. Genet. Devel.* 7, 75; Marais, 1996, *Cancer Surveys* 27; Naumann, 1997, *Cancer Res.* 143, 237). c-Raf is the cellular homolog of v-Raf, the transforming element of the murine sarcoma virus 3611. The Raf family consists of three highly 15 conserved isozymes in vertebrates: c-Raf-1, which is constitutively expressed in all tissues, A-Raf, which is expressed in urogenital tissue and B-Raf which is expressed in and cerebrum and testes (Storm, 1990, *Oncogene* 5, 345). Inappropriate expression of these key genes involved in cell growth and differentiation can result in uncontrolled cell proliferation and/or propagation of damaged DNA, leading to hyperproliferative disorders 20 such as cancer, restenosis, psoriasis and rheumatoid arthritis.

25 Raf is one of the major downstream effectors of Ras, a member of the class of small GDP/GTP-binding proteins involved in cellular signal transduction pathways (figure 35; Marshall, 1995, *Molec. Reprod. Devel.*, 42, 493). Appropriate mitogenic signals cause an increase in levels of the GTP-bound Ras. In its GTP-bound active state, Ras binds Raf and localizes it to the plasma membrane. This results in activation of the Raf kinase activity. Activated Raf in turn phosphorylates MEK, thereby activating the

MAP kinase signaling cascade leading to cell cycle progression. Amino terminal truncation of Raf leads to constitutively active protein. Expression of either constitutively active Raf or constitutively active MEK is sufficient for oncogenic transformation of fibroblasts (Cowley, 1994, *Cell* 77, 81; Mansour, 1994, *Science* 265, 966; Kolch, 1991, 5 *Nature* 349, 426). In normal cells, the expression level of Raf is limiting in cellular transformation (Cuadrado, 1993, *Oncogene* 8, 2443). The pivotal position that the Ras and Raf family of proteins occupy in cellular signal transduction pathways emphasizes their importance in the control of normal cellular growth.

Activation of Raf in mammalian cells is triggered by a variety of growth factors 10 and cytokines. Raf activation has been observed in cardiac myocyte cultures stimulated by fibroblast growth factor (FGF), endothelin or phorbol ester (Bogoyevitch, 1995, *J. Biol. Chem.* 270, 1). Activation has also been seen in Swiss 3T3 cells treated with bombesin and platelet derived growth factor (Mitchell, 1995, *J. Biol. Chem.* 270, 8623) or with colony stimulating factor or lipopolysaccharide (Reimann, 1994, *J. Immun.* 153, 398), 15 in L6 myoblasts stimulated with insulin-like growth factor (Cross, 1994, *Biochem J.* 303, 21), as well as in B cells stimulated via the immunoglobulin receptor (Kumar, 1995, *Biochem J.* 307, 215).

There is growing evidence from a number of laboratories that suggests that the 20 Ras/Raf pathway may also be involved in cell motility (Bar-Sagi and Feramisco, 1986 *Science* 233, 1061; Partin *et al.*, 1988 *Cancer Res.* 48-6050; Fox *et al.*, 1994 *Oncogene* 9, 3519). These studies show that cell lines transfected with activated Ras show an increase in ruffling, pseudopod extension and chemotactic response, all of which are cell-motility-related processes. Uncontrolled cell motility has been implicated in several pathological processes such as restenosis, angiogenesis and wound healing.

25 Raf activation leads to induction of several immediate early transcription factors including NF- $\kappa$ B and AP-1 (Bruder, 1992, *Genes Devel.* 6, 545; Finco, 1993, *J. Biol. Chem.* 268, 17676). AP-1 regulates expression of a variety of proteases (Sato, 1994 *Oncogene* 8, 395; Gaire, 1994, *J. Biol. Chem.* 269, 2032; Lauricell-Lefebvre, 1993,

5 *Invasion Metastasis* 13, 289; Troen, 1991, *Cell Growth Differ* 2, 23). A cascade of MMP and serine proteinase expression is implicated in the acquisition of an invasive phenotype as well as in angiogenesis in tumors (MacDougall, 1995, *Cancer and Metastasis Reviews* 14, 351). Thus, Raf signaling is expected to contribute to increased invasiveness in tumor cells, leading to metastasis.

10 Coexpression studies of Raf-1 and Bcl-2 have shown that these proteins bind and interact to synergistically suppress apoptosis (Wang, 1994, *Oncogene* 9, 2751). Thus, overexpression of Raf-1 in tumor cells is likely to contribute to malignant transformation and increased resistance to chemotherapeutic agents. Overexpression of c-Raf-1 is observed in squamous cell carcinomas of the head and neck taken from patients resistant to radiation therapy (Riva, 1995, *Oral Oncol., Eur. J. Cancer* 31B, 384) and in lung carcinomas (Rapp, 1988, *The Oncogene Handbook*, 213). Activated (truncated) Raf has been detected in a number of human cancers including small-cell lung, stomach, renal, breast and laryngeal cancer (Rapp, 1988, *The Oncogene Handbook*, 213).

15 Therapeutic intervention in down-regulating Raf expression have focused on antisense oligonucleotide approaches:

20 Antisense oligonucleotides targeting c-Raf-1 were used to demonstrate that IL-2 stimulated growth of T cells requires c-raf (Riedel, 1993, *Eur. J. Immunol.* 23, 3146). Antisense oligonucleotides targeting c-Raf-1 in SQ-20B cells showed reduced Raf expression and increased radiation sensitivity (Soldatenkov, 1997, *The Cancer J. from Scientific American* 3, 13). Rapp et al. have disclosed a method for inhibiting c-Raf-1 gene expression using a vector expressing the gene in the antisense orientation (International PCT Publication No. WO 93/04170). Antisense oligonucleotides targeting c-Raf-1 in SQ-20B cells showed reduced DNA synthesis in response to insulin 25 stimulation in rat hepatoma cells (Tornkvist, 1994, *J. Biol. Chem.* 269, 13919). Monia et al. have disclosed a method for inhibiting Raf expression using antisense oligonucleotides (U.S. Patent No. 5,563,255) and shown that antisense oligonucleotides targeting c-Raf-1 can inhibit Raf mRNA expression in cell culture, and inhibit growth of a variety of tumor

types in human tumor xenograft models (Monia *et al.*, 1996, *Proc. Natl. Acad. Sci.* 93, 15481; Monia *et al.*, 1996, *Nature Med.* 2, 668). No toxicity was observed in these studies following systemic administration of c-Raf antisense oligonucleotides, suggesting that at least partial down regulation of Raf in normal tissues is not overtly toxic.

5 It has been proposed that synthetic ribozymes can be delivered to target cells exogenously in the presence or absence of lipid delivery vehicles (Thompson *et al.*, International PCT Publication No. WO 93/23057; Sullivan *et al.*, International PCT Publication No. WO 94/02595).

Recently Sandberg *et al.*, 1996, Abstract, IBC USA Conferences on Angiogenesis 10 Inhibitors and other novel therapeutics for Ocular Diseases of Neovascularization, reported pharmacokinetics of a chemically modified hammerhead ribozyme targeted against a vascular endothelial growth factor (VEGF) receptor RNA in normal and tumor bearing mice after daily bolus or continuous infusion.

Desjardins *et al.*, 1996, *J. Pharmacol. Exptl. Therapeutic*, 27, 8, 1419, reported 15 pharmacokinetics of a synthetic, chemically modified hammerhead ribozyme against the rat cytochrome P-450 3A2 mRNA after single intravenous injection.

The references cited above are distinct from the presently claimed invention since they do not disclose and/or contemplate the use of ribozymes to cleave Raf RNA. Furthermore, Applicant believes that the references do not disclose and/or enable the use 20 of ribozymes to down regulate normal Raf gene expression in mammalian cells and/or whole animal.

#### Summary Of The Invention

This invention relates to identification, synthesis and use of nucleic acid catalysts to cleave RNA species that are required for cellular growth responses. In particular, 25 applicant describes the selection and function of ribozymes capable of cleaving RNA

encoded by *c-raf* gene. Such ribozymes may be used to inhibit the hyper-proliferation of tumor cells in one or more cancers, restenosis, psoriasis, fibrosis and rheumatoid arthritis.

In the present invention, ribozymes that cleave *c-raf* RNA are described. Moreover, applicant shows that these ribozymes are able to inhibit gene expression and cell proliferation *in vitro* and *in vivo*, and that the catalytic activity of the ribozymes is required for their inhibitory effect. From those of ordinary skill in the art, it is clear from the examples described herein, that other ribozymes that cleave target RNAs required for cell proliferation may be readily designed and are within the invention.

By "inhibit" is meant that the activity of *c-raf* or level of RNAs encoded by *c-raf* is reduced below that observed in the absence of the nucleic acid, particularly, inhibition with ribozymes is preferably below that level observed in the presence of an inactive RNA molecule able to bind to the same site on the mRNA, but unable to cleave that RNA.

By "nucleic acid catalyst" is meant a nucleic acid molecule capable of catalyzing (altering the velocity and/or rate of) a variety of reactions including the ability to repeatedly cleave other separate nucleic acid molecules (endonuclease activity) in a nucleotide base sequence-specific manner. Such a molecule with endonuclease activity may have complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity that specifically cleaves RNA or DNA in that target. That is, the nucleic acid molecule with endonuclease activity is able to intramolecularly or intermolecularly cleave RNA or DNA and thereby inactivate a target RNA or DNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA or DNA to allow the cleavage to occur. 100% complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention. The nucleic acids may be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, catalytic oligonucleotides, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease,

endonuclease, minizyme, leadzyme, oligozyme or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity. The specific nucleic acid catalysts described in the instant application are not limiting in the invention and those skilled in the art will recognize that all that is important in a nucleic acid catalyst of this invention is that it has a specific substrate binding site which is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart a nucleic acid cleaving activity to the molecule.

By "substrate binding arm" or "substrate binding domain" is meant that portion/region of a ribozyme which is complementary to (*i.e.*, able to base-pair with) a portion of its substrate. Generally, such complementarity is 100%, but can be less if desired. For example, as few as 10 bases out of 14 may be base-paired. Such arms are shown generally in Figure 1 and 3. That is, these arms contain sequences within a ribozyme which are intended to bring ribozyme and target RNA together through complementary base-pairing interactions. The ribozyme of the invention may have binding arms that are contiguous or non-contiguous and may be of varying lengths. The length of the binding arm(s) are preferably greater than or equal to four nucleotides; specifically 12-100 nucleotides; more specifically 14-24 nucleotides long. If two binding arms are chosen, the design is such that the length of the binding arms are symmetrical (*i.e.*, each of the binding arms is of the same length; *e.g.*, five and five nucleotides, six and six nucleotides or seven and seven nucleotides long) or asymmetrical (*i.e.*, the binding arms are of different length; *e.g.*, six and three nucleotides; three and six nucleotides long; four and five nucleotides long; four and six nucleotides long; four and seven nucleotides long; and the like).

In one of the preferred embodiments of the inventions herein, the nucleic acid catalyst is formed in a hammerhead or hairpin motif, but may also be formed in the motif of a hepatitis d virus, group I intron, group II intron or RNaseP RNA (in association with an RNA guide sequence) or *Neurospora* VS RNA. Examples of such hammerhead motifs

are described by Dreyfus, *supra*, Rossi *et al.*, 1992, *AIDS Research and Human Retroviruses* 8, 183; of hairpin motifs by Hampel *et al.*, EP0360257, Hampel and Tritz, 1989 *Biochemistry* 28, 4929, Feldstein *et al.*, 1989, *Gene* 82, 53, Haseloff and Gerlach, 1989, *Gene*, 82, 43, and Hampel *et al.*, 1990 *Nucleic Acids Res.* 18, 299; of the hepatitis d 5 virus motif is described by Perrotta and Been, 1992 *Biochemistry* 31, 16; of the RNaseP motif by Guerrier-Takada *et al.*, 1983 *Cell* 35, 849; Forster and Altman, 1990, *Science* 249, 783; Li and Altman, 1996, *Nucleic Acids Res.* 24, 835; *Neurospora* VS RNA 10 ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696; Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Collins and Olive, 1993 *Biochemistry* 32, 2795-2799; Guo and Collins, 1995, *EMBO. J.* 14, 363); Group II 15 introns are described by Griffin *et al.*, 1995, *Chem. Biol.* 2, 761; Michels and Pyle, 1995, *Biochemistry* 34, 2965; Pyle *et al.*, International PCT Publication No. WO 96/22689; and of the Group I intron by Cech *et al.*, U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in a nucleic acid catalyst of this invention is that it has a specific substrate 20 binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

By "equivalent" RNA to *c-raf* is meant to include those naturally occurring RNA 25 molecules associated with cancer in various animals, including human, rodent, primate, rabbit and pig. The equivalent RNA sequence also includes in addition to the coding region, regions such as 5'-untranslated region, 3'-untranslated region, introns, intron-exon junction and the like.

By "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with 25 another RNA sequence by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-paired interactions.

In a preferred embodiment the invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high degree of specificity for the RNA of a

desired target. The nucleic acid catalyst is preferably targeted to a highly conserved sequence region of a target mRNAs encoding c-raf proteins such that specific treatment of a disease or condition can be provided with either one or several enzymatic nucleic acids. Such nucleic acid catalysts can be delivered exogenously to specific cells as required.

5     Alternatively, the ribozymes can be expressed from DNA/RNA vectors that are delivered to specific cells.

Such ribozymes are useful for the prevention of the diseases and conditions discussed above, and any other diseases or conditions that are related to the levels of c-Raf activity in a cell or tissue.

10     By "related" is meant that the inhibition of c-raf RNAs and thus reduction in the level of respective protein activity will relieve to some extent the symptoms of the disease or condition.

15     In preferred embodiments, the ribozymes have binding arms which are complementary to the target sequences in Tables XII-XIX. Examples of such ribozymes are also shown in Tables XII-XIX. Examples of such ribozymes consist essentially of sequences defined in these Tables.

20     By "consists essentially of" is meant that the active ribozyme contains an enzymatic center or core equivalent to those in the examples, and binding arms able to bind mRNA such that cleavage at the target site occurs. Other sequences may be present which do not interfere with such cleavage.

Thus, in a first aspect, the invention features ribozymes that inhibit gene expression and/or cell proliferation. These chemically or enzymatically synthesized RNA molecules contain substrate binding domains that bind to accessible regions of their target mRNAs. The RNA molecules also contain domains that catalyze the cleavage of RNA. The RNA molecules are preferably ribozymes of the hammerhead or hairpin motif. Upon binding, the ribozymes cleave the target mRNAs, preventing translation and protein accumulation. In the absence of the expression of the target gene, cell proliferation is inhibited.

In a preferred embodiment, ribozymes are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues ex vivo, or in vivo through injection, infusion pump or stent, with or without their incorporation in biopolymers. In another preferred embodiment, the ribozyme is administered to the site of *c-ras* expression (e.g., tumor cells) in an appropriate liposomal vehicle.

In another aspect of the invention, ribozymes that cleave target molecules and inhibit *c-ras* activity are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the ribozymes are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of ribozymes. Such vectors might be repeatedly administered as necessary. Once expressed, the ribozymes cleave the target mRNA. Delivery of ribozyme expressing vectors could be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture and Stinchcomb, 1996, *TIG.*, 12, 510). In another aspect of the invention, ribozymes that cleave target molecules and inhibit cell proliferation are expressed from transcription units inserted into DNA, RNA, or viral vectors. Preferably, the recombinant vectors capable of expressing the ribozymes are locally delivered as described above, and transiently persist in smooth muscle cells. 25 However, other mammalian cell vectors that direct the expression of RNA may be used for this purpose.

By "patient" is meant an organism which is a donor or recipient of explanted cells or the cells themselves. "Patient" also refers to an organism to which nucleic acid

catalysts can be administered. Preferably, a patient is a mammal or mammalian cells. More preferably, a patient is a human or human cells.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

5        These ribozymes, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed above. For example, to treat a disease or condition associated with c-raf levels, the patient may be treated, or other appropriate cells may be treated, as is evident to those skilled in the art.

10      In a further embodiment, the described ribozymes can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described ribozymes could be used in combination with one or more known therapeutic agents to treat cancer.

15      In preferred embodiments, the ribozymes have binding arms which are complementary to the sequences in the tables, shown as Seq. I.D. Nos. 1-501, 1078-1152, 1461-1768, 1841-1912, 2354-2794 and 2846-2956. Examples of such ribozymes are shown as Seq. I.D. Nos. 502-1002, 1003-1077, 1153-1460, 1769-1840, 1913-2353 and 2795-2845. Other sequences may be present which do not interfere with such cleavage.

20      Ribozymes that cleave the specified sites in Raf mRNAs represent a novel therapeutic approach to treat tumor angiogenesis, ocular diseases, rheumatoid arthritis, psoriasis and others. Applicant indicates that ribozymes are able to inhibit the activity of Raf and that the catalytic activity of the ribozymes is required for their inhibitory effect. Those of ordinary skill in the art will find that it is clear from the examples described that other ribozymes that cleave Raf mRNAs may be readily designed and are within the invention.

25      Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description Of The Preferred Embodiments

The drawings will first briefly be described.

Drawings:

Figure 1 shows the secondary structure model for seven different classes of nucleic acid catalysts. Arrow indicates the site of cleavage. ----- indicate the target sequence. Lines interspersed with dots are meant to indicate tertiary interactions. - is meant to indicate base-paired interaction. **Group I Intron:** P1-P9.0 represent various stem-loop structures (Cech *et al.*, 1994, *Nature Struc. Bio.*, 1, 273). **RNase P (M1RNA):** EGS represents external guide sequence (Forster *et al.*, 1990, *Science*, 249, 783; Pace *et al.*, 1990, *J. Biol. Chem.*, 265, 3587). **Group II Intron:** 5'SS means 5' splice site; 3'SS means 3'-splice site; IBS means intron binding site; EBS means exon binding site (Pyle *et al.*, 1994, *Biochemistry*, 33, 2716). **VS RNA:** I-VI are meant to indicate six stem-loop structures; shaded regions are meant to indicate tertiary interaction (Collins, International PCT Publication No. WO 96/19577). **HDV Ribozyme:** I-IV are meant to indicate four stem-loop structures (Been *et al.*, US Patent No. 5,625,047). **Hammerhead Ribozyme:** I-III are meant to indicate three stem-loop structures; stems I-III can be of any length and may be symmetrical or asymmetrical (Usman *et al.*, 1996, *Curr. Op. Struct. Bio.*, 1, 527). **Hairpin Ribozyme:** Helix 1, 4 and 5 can be of any length; Helix 2 is between 3 and 8 base-pairs long; Y is a pyrimidine; Helix 2 (H2) is provided with a least 4 base pairs (*i.e.*, n is 1, 2, 3 or 4) and helix 5 can be optionally provided of length 2 or more bases (preferably 3 - 20 bases, *i.e.*, m is from 1 - 20 or more). Helix 2 and helix 5 may be covalently linked by one or more bases (*i.e.*, r is 1 base). Helix 1, 4 or 5 may also be extended by 2 or more base pairs (*e.g.*, 4 - 20 base pairs) to stabilize the ribozyme structure, and preferably is a protein binding site. In each instance, each N and N' independently is any normal or modified base and each dash represents a potential base-pairing interaction. These nucleotides may be modified at the sugar, base or phosphate. Complete base-pairing is not required in the helices, but is preferred. Helix 1 and 4 can be of any size (*i.e.*, o and p is each independently from 0 to any number, *e.g.*, 20) as long

as some base-pairing is maintained. Essential bases are shown as specific bases in the structure, but those in the art will recognize that one or more may be modified chemically (abasic, base, sugar and/or phosphate modifications) or replaced with another base without significant effect. Helix 4 can be formed from two separate molecules, *i.e.*, 5 without a connecting loop. The connecting loop when present may be a ribonucleotide with or without modifications to its base, sugar or phosphate. "q" is 2 bases. The connecting loop can also be replaced with a non-nucleotide linker molecule. H refers to bases A, U, or C. Y refers to pyrimidine bases. "—" refers to a covalent bond. (Burke *et al.*, 1996, *Nucleic Acids & Mol. Biol.*, 10, 129; Chowtira *et al.*, US Patent No. 10 5,631,359).

Figure 2 shows a general approach to accessible site and target discovery using nucleic acid catalysts.

Figure 3 is a diagram of a hammerhead ribozyme. The consensus hammerhead cleavage site in a target RNA is a "U" followed by "H" (anything but "G"). The 15 hammerhead ribozyme cleaves after the "H." This simple di-nucleotide sequence occurs, on average, every 5 nt in a target RNA. Thus, there are approximately 400 potential hammerhead cleavage sites in a 2-Kb mRNA. Stems I and III are formed by hybridization of the hammerhead binding arms with the complementary sequence in target RNA; it is these binding arms that confer specificity to the hammerhead ribozyme 20 for its target. The binding arms of the hammerhead are interrupted by the catalytic domain that forms part of the structure responsible for cleavage.

Figure 4 shows a scheme for the design and synthesis of a Defined Library: simultaneous screen of 400 different ICAM-targeted ribozymes is used as an example. 25 DNA oligonucleotides encoding each ICAM-targeted ribozyme are synthesized individually (A), pooled (B), then cloned and converted to retroviral vectors as a pool. The resulting retroviral vector particles are used to transduce a target cell line that expresses ICAM (B). Cells expressing ribozymes that inhibit ICAM expression (ICAM-low) are sorted from cells expressing ineffective ribozymes by FACS sorting (C),

effective ribozymes enriched in the ICAM-low population of cells are identified by filter hybridization (D).

Figure 5 A) shows randomization of the binding arms of a hammerhead ribozyme to produce a Random Library. The binding arms can be of any length and any symmetry, 5 *i.e.*, symmetrical or assymmetrical. B) shows complexities of hammerhead Random Ribozyme Libraries comprising a 6-nt or a 7-nt long binding arms.

Figure 6 is a schematic overview of Target Discovery strategy. An oligonucleotide is prepared in a single reaction vessel in which all 4 standard nucleotides are incorporated in a random fashion in the target binding arm(s) of the ribozyme to 10 produce a pool of all possible ribozymes (A). This pool is cloned into an appropriate vector in a single tube to produce the Random Library expression vector (B) and retroviral vector particles are produced from this pool in a single tube (C). The resulting Random Ribozyme Library retroviral expression vector pool is then used to transduce a cell type of interest (D). Cells exhibiting the desired phenotype are then separated from 15 the rest of the population using a number of possible selection strategies (E and see text). Genes that are critical for expression of the selected phenotype can then be identified by sequencing the target binding arms of ribozymes contained in the selected population (F).

Figure 7 shows an example of application of Random Ribozyme Libraries to identify genes critical for the induction of ICAM expression. Human Umbilical Vein 20 Endothelial Cells (HUVECs) are transduced with a Random Ribozyme Library (A), ICAM expression is induced using TNF-alpha (B), and cells expressing ribozymes that inhibit ICAM induction are selected from cells expressing ineffective ribozymes by sorting ICAM-low cells (C). Genes critical for ICAM induction are identified by 25 sequencing the binding arms of the ribozymes that inhibit ICAM expression in the ICAM-low cells.

Figure 8 is an example of an efficient cloning strategy for producing a Defined or Random Ribozyme Libraries. DNA oligos encoding ribozyme coding regions and

restriction sites for cloning are designed to also contain a stem-loop structure on the 3' ends (1). This stem loop forms an intramolecular primer site for extension to form a double-stranded molecule by DNA polymerase (2). The double-stranded fragment is cleaved with appropriate restriction endonucleases to produce suitable ends for 5 subsequent cloning (3).

Figure 9 shows molecular analysis of the PNP-targeted Defined Ribozyme Library: sequence analysis. Plasmid DNA from the PNP-targeted Defined Ribozyme Library was prepared and sequenced as a pool. The sequencing primer used reads the non-coding strand of the region encoding the ribozymes. Note that the sequence diverges 10 at the binding arm, converges at the catalytic domain (5' - TTTCGGCCTAACGGCCTCATCAG-3'), and then diverges at the other binding arm. These results are consistent with those expected for a sequence of a heterogeneous pool of clones containing different sequences at the ribozyme binding arms.

Figure 10 shows molecular analysis of the PNP-targeted Defined Ribozyme Library: sequence analysis after propagation in Sup T1 human T cells and selection in 15 10 mmol 6-thioguanosine. Sup T1 cells were transduced with retroviral vector-based Defined Ribozyme Library comprised of 40 different PNP-targeted ribozyme oligos cloned into the U6+27 transcription unit (Figure 11D). The cells were propagated for 2 weeks following transduction, then subjected to 16 days of selection in 10 mmol 6-thioguanosine. Surviving cells were harvested, and ribozyme sequences present in the 20 selected population of cells were amplified and sequenced. Note that, relative to the original Library where sequences of the binding arms were ambiguous due to the presence of 40 different ribozymes (Figure 9), the sequence of the binding arms in the selected population corresponded to only 1 of the 40 ribozymes included in the Library. These 25 results suggest that this ribozyme was the most-potent ribozyme of 40 ribozymes tested.

Figure 11 is a schematic representation of transcription units suitable for expression ribozyme library of the instant invention. A) is a diagrammatic representation of some RNA polymerase (Pol) II and III ribozyme (RZ) transcription units. CMV

Promoter Driven is a Pol II transcript driven by a cytomegalovirus promoter; the transcript can be designed such that the ribozyme is at the 5'-region, 3'-region or somewhere in between and the transcript optionally comprises an intron. tRNA-DC is a Pol III transcript driven by a transfer RNA (tRNA) promoter, wherein the ribozyme is at the 3'-end of the transcript; the transcript optionally comprises a stem-loop structure 3' of the ribozyme. U6+27 is a Pol III transcript driven by a U6 small nuclear (snRNA) promoter; ribozyme is 3' of a sequence that is homologous to 27 nucleotides at the 5'-end of a U6 snRNA; the transcript optionally comprises a stem-loop structure at the 3'-end of the ribozyme. VAI-90 is a Pol III transcript driven by an adenovirus VA promoter; ribozyme is 3' of a sequence homologous to 90 nucleotides at the 5'-end of a VAI RNA; the transcript optionally comprises a stem-loop structure at the 3'-end of the ribozyme. VAC is a Pol III transcript driven by an adenovirus VAI promoter; the ribozyme is inserted towards the 3'-region of the VA RNA and into a S35 motif, which is a stable greater than or equal to 8 bp long intramolecular stem formed by base-paired interaction between sequences in the 5'-region and the 3'-region flanking the ribozyme (see Beigelman *et al.*, International PCT Application No. WO 96/18736); the S35 domain positions the ribozyme away from the main transcript as an independent domain. TRZ is a Pol III transcript driven by a tRNA promoter; ribozyme is inserted in the S35 domain and is positioned away from the main transcript (see Beigelman *et al.*, International PCT Application No. WO 96/18736). B) shows various transcription units based on the U1 small nuclear RNA (snRNA) system. C) is a schematic representation of a retroviral vectors encoding ribozyme genes. NGFR, nerve growth factor receptor is used as a selectable marker, LTR, long terminal repeat of a retrovirus, UTR, untranslated region. D) shows a U6+27 hammerhead ribozyme transcription unit based on the U6 snRNA. The ribozyme transcript comprises the first 27 nt from the U6 snRNA which is reported to be necessary for the stability of the transcript. The transcript terminates with a stretch of uridine residues. The hammerhead ribozyme shown in the figure has random (N) binding arm sequence.

Figure 12 is a schematic representation of a combinatorial approach to the screening of ribozyme variants.

Figure 13 shows the sequence of a Starting Ribozyme to be used in the screening approach described in Figure 12. The Starting Ribozyme is a hammerhead (HH) 5 ribozyme designed to cleave target RNA A (HH-A). Position 7 in HH-A is also referred to in this application as position 24 to indicate that U24 is the 24th nucleotide incorporated into the HH-A ribozyme during chemical synthesis. Similarly, positions 4 and 3 are also referred to as positions 27 and 28, respectively. s indicates phosphorothioate substitution. Lower case alphabets in the HH-A sequence indicate 2'-10 O-methyl nucleotides; uppercase alphabets in the sequence of HH-A at positions 5, 6, 8, 12 and 15.1 indicate ribonucleotides. Positions 3, 4 and 7 are shown as uppercase, large alphabets to indicate the positions selected for screening using the method shown in Figure 12. ● indicates base-paired interaction. iB represents abasic inverted deoxyribose moiety.

15 Figure 14 shows a scheme for screening variants of HH-A ribozyme. Positions 24, 27 and 28 are selected for analysis in this scheme.

Figure 15 shows non-limiting examples of some of the nucleotide analogs that can be used to construct ribozyme libraries. 2'-O-MTM-U represents 2'-O-methylthiomethyl uridine; 2'-O-MTM-C represents 2'-O-methylthiomethyl cytidine; 6-Me-U represents 6-methyl uridine (Beigelman *et al.*, International PCT Publication No. WO 96/18736 which is incorporated by reference herein).

20 Figure 16 shows activity of HH-A variant ribozymes as determined in a cell-based assay. \* indicates the substitution that provided the most desirable attribute in a ribozyme.

25 Figure 17A shows the sequence and chemical composition of ribozymes that showed the most desirable attribute in a cell.

Figure 17B shows formulae for four different novel ribozyme motifs.

Figure 18 shows the formula for a novel ribozyme motif.

Figure 19 shows the sequence of a Starting Ribozyme to be used in the screening approach described in Figure 14. A HH ribozyme targeted against RNA B (HH-B) was 5 chosen for analysis of the loop II sequence variants.

Figure 20 shows a scheme for screening loop-II sequence variants of HH-B ribozyme.

Figure 21 shows the relative catalytic rates ( $k_{rel}$ ) for RNA cleavage reactions catalyzed by HH-B loop-II variant ribozymes.

10 Figure 22 is a schematic representation of HH-B ribozyme-substrate complex and the activity of HH-B ribozyme with either the 5'-GAAA-3' or the 5'-GUUA-3' loop-II sequence.

Figure 23 shows a scheme for using a combinatorial approach to identify potential ribozyme targets by varying the binding arms.

15 Figure 24 shows a scheme for using a combinatorial approach to identify novel ribozymes by the varying putative catalytic domain sequence.

Figure 25 shows a table of accessible sites within a Bcl-2 transcript ((975 nucleotides) which were found using the combinatorial in vitro screening process.

20 Figure 26 shows a table of accessible sites with a Kras transcript (796 nucleotides) which were found using the combinatorial in vitro screening process as well as a graphic depiction of relative activity of ribozymes to those sites.

Figure 27 shows a table of accessible sites with a UPA transcript (400 nucleotides) which were found using the combinatorial in vitro screening process as well as a graphic depiction of relative activity of ribozymes to those sites.

Figure 28 shows a graph displaying data from a ribonuclease protection assay (RPA) after treatment of MCF-7 cells with ribozymes targeted to site 549 of the transcript (Seq.ID #9). The Bcl-2 mRNA isolated from MCF-7 cells is normalized to GAPDH which was also probed in the RPA. The graph includes an untreated control and 5 an irrelevant ribozyme (no complementarity with Bcl-2 mRNA).

Figure 29 displays a schematic representation of NTP synthesis using nucleoside substrates.

Figure 30 depicts a scheme for the synthesis of a xylo ribonucleoside phosphoramidite.

10 Figure 31 is a diagrammatic representation of hammerhead (HH) ribozyme targeted against stromelysin RNA (site 617) with various modifications.

Figure 32 is a schematic representation of a one pot deprotection of RNA synthesized using RNA phosphoramidite chemistry.

15 Figure 33 is a comparison of a one-pot and a two-pot process for deprotection of RNA.

Figure 34 shows the results of a one-pot deprotection with different polar organic reagents.

Figure 35 is a diagrammatic representation of ras signal transduction pathway.

20 Figure 36 is a diagrammatic representation of hammerhead ribozymes targeted against c-raf RNA.

Figure 37 is a graphical representation of c-raf 2'-C-allyl 1120 hammerhead (HH) ribozyme-mediated inhibition of cell proliferation.

Figure 38 is a graphical representation of inhibition of cell proliferation mediated by c-raf 2'-C-allyl 1120 and 1251 hammerhead (HH) ribozymes.

Figure 39 shows the effects of *flt-1* ribozymes (active/inactive) on LLC-HM primary tumor growth in mice.

Figure 40 shows the effects of *flt-1* ribozymes on LLC-HM primary tumor volume immediately following the cessation of treatment.

5 Figure 41 shows the effects of *flt-1* ribozymes on lung metastatic indices (number of metastases and lung mass).

Figure 42 shows the effects of *flk-1* ribozymes (active/inactive) on LLC-HM primary tumor growth in mice.

10 Figure 43 shows the effects of *flk-1* ribozymes on LLC-HM primary tumor volume immediately following the cessation of treatment.

Figure 44 shows the effects of *flk-1* ribozymes on lung metastatic indices (number of metastases and lung mass).

Nucleic Acid Catalysts:

15 Catalytic nucleic acid molecules (ribozymes) are nucleic acid molecules capable of catalyzing one or more of a variety of reactions, including the ability to repeatedly cleave other separate nucleic acid molecules in a nucleotide base sequence-specific manner. Such nucleic acid catalysts can be used, for example, to target cleavage of virtually any RNA transcript (Zaug *et al.*, 324, *Nature* 429 1986 ; Cech, 260 *JAMA* 3030, 20 1988; and Jefferies *et al.*, 17 *Nucleic Acids Research* 1371, 1989). Catalytic nucleic acid molecules mean any nucleotide base-comprising molecule having the ability to repeatedly act on one or more types of molecules, including but not limited to nucleic acid catalysts. By way of example but not limitation, such molecules include those that are able to repeatedly cleave nucleic acid molecules, peptides, or other polymers, and those that are 25 able to cause the polymerization of such nucleic acids and other polymers. Specifically, such molecules include ribozymes, DNAzymes, external guide sequences and the like. It

is expected that such molecules will also include modified nucleotides compared to standard nucleotides found in DNA and RNA.

Because of their sequence-specificity, *trans*-cleaving nucleic acid catalysts show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Nucleic acid catalysts can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the RNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively inhibited. In addition, nucleic acid catalysts can be used to validate a therapeutic gene target and/or to determine the function of a gene in a biological system (Christoffersen, 1997, *Nature Biotech.* 15, 483).

There are at least seven basic varieties of enzymatic RNA molecules derived from naturally occurring self-cleaving RNAs (see Table I). Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a substrate/target RNA. Such binding occurs through the substrate/target binding portion of an nucleic acid catalyst which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic and selective cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and thus can repeatedly bind and cleave new targets.

In addition, several *in vitro* selection (evolution) strategies (Orgel, 1979, *Proc. R. Soc. London, B* 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing a variety of reactions, such as cleavage and ligation of phosphodiester linkages and amide linkages, (Joyce, 1989, *Gene*, 82, 83-87; Beaudry *et al.*, 1992, *Science* 257,

635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.*, 1994, *TIBTECH* 12, 268; Bartel *et al.*, 1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.*, 1995, *FASEB J.*, 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442; Breaker, 1997, *Nature Biotech.* 15, 427).

5 There are several reports that describe the use of a variety of *in vitro* and *in vivo* selection strategies to study structure and function of catalytic nucleic acid molecules (Campbell *et al.*, 1995, *RNA* 1, 598; Joyce 1989, *Gene*, 82,83; Lieber *et al.*, 1995, *Mol Cell Biol.* 15, 540; Lieber *et al.*, International PCT Publication No. WO 96/01314; Szostak 1988, in *Redesigning the Molecules of Life*, Ed. S. A. Benner, pp 87, Springer-  
10 Verlag, Germany; Kramer *et al.*, U.S. Patent No. 5,616,459; Draper *et al.*, US Patent No. 5,496,698; Joyce, U.S. Patent No. 5,595,873; Szostak *et al.*, U.S. Patent No. 5,631,146).

15 The enzymatic nature of a ribozyme is advantageous over other technologies, since the effective concentration of ribozyme sufficient to effect a therapeutic treatment is generally lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme (enzymatic nucleic acid) molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base-pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base-pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

20 25 The development of ribozymes that are optimal for catalytic activity would contribute significantly to any strategy that employs RNA-cleaving ribozymes for the purpose of regulating gene expression. The hammerhead ribozyme, for example, functions with a catalytic rate ( $k_{cat}$ ) of  $\sim 1 \text{ min}^{-1}$  in the presence of saturating (10 mM)

concentrations of Mg<sup>2+</sup> cofactor. However, the rate for this ribozyme in Mg<sup>2+</sup> concentrations that are closer to those found inside cells (0.5 - 2 mM) can be 10- to 100-fold slower. In contrast, the RNase P holoenzyme can catalyze pre-tRNA cleavage with a  $k_{cat}$  of ~30 min<sup>-1</sup> under optimal assay conditions. An artificial 'RNA ligase' ribozyme 5 (Bartel *et al.*, *supra*) has been shown to catalyze the corresponding self-modification reaction with a rate of ~100 min<sup>-1</sup>. In addition, it is known that certain modified hammerhead ribozymes that have substrate binding arms made of DNA catalyze RNA cleavage with multiple turn-over rates that approach 100 min<sup>-1</sup>. Finally, replacement of a specific residue within the catalytic core of the hammerhead with certain nucleotide 10 analogues gives modified ribozymes that show as much as a 10-fold improvement in catalytic rate. These findings demonstrate that ribozymes can promote chemical transformations with catalytic rates that are significantly greater than those displayed *in vitro* by most natural self-cleaving ribozymes. It is then possible that the structures of 15 certain self-cleaving ribozymes may not be optimized to give maximal catalytic activity, or that entirely new RNA motifs could be made that display significantly faster rates for RNA phosphoester cleavage.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a sugar moiety. Nucleotide generally comprise a base, sugar and a 20 phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other ; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; all 25 hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art and has recently been summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into enzymatic nucleic acids without significantly effecting their catalytic activity include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl,

5 pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g., 6-methyluridine) and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases may be used within the catalytic core of the enzyme and/or in the substrate-binding regions.

10 In another preferred embodiment, catalytic activity of the molecules described in the instant invention can be optimized as described by Draper *et al.*, *supra*. The details will not be repeated here, but include altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases and/or enhance their enzymatic activity (see e.g., Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken *et al.*, 1991 *Science* 253, 314; Usman and Cedergren, 1992 *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al.*, *supra*; all of these describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of enzymatic RNA molecules). Modifications which enhance their efficacy in cells, and removal of bases from stem loop structures to shorten RNA synthesis times and reduce chemical requirements are desired. (All of these publications are hereby incorporated by reference herein).

25 There are several examples in the art describing sugar and phosphate modifications that can be introduced into nucleic acid catalysts without significantly effecting catalysis and with significant enhancement in their nuclease stability and efficacy. Ribozymes are modified to enhance stability and/or enhance catalytic activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-flouro, 2'-O-methyl, 2'-H, nucleotide base modifications (for a review see Usman and

Cedergren, 1992 *TIBS* 17, 34; Usman *et al.*, 1994 *Nucleic Acids Symp. Ser.* 31, 153; Burgin *et al.*, 1996 *Biochemistry* 35, 14090). Sugar modification of nucleic acid catalysts has been extensively described in the art (see Eckstein *et al.*, *International Publication PCT No. WO 92/07065*; Perrault *et al.* *Nature* 1990, 344, 565-568; Pieken *et al.* *Science* 1991, 253, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* 1992, 17, 334-339; 5 Usman *et al.* *International Publication PCT No. WO 93/15187*; Sproat, *US Patent No. 5,334,711* and Beigelman *et al.*, 1995 *J. Biol. Chem.* 270, 25702; all of the references are hereby incorporated in their totality by reference herein).

Such publications describe general methods and strategies to determine the 10 location of incorporation of sugar, base and/or phosphate modifications and the like into ribozymes without inhibiting catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the nucleic acid catalysts of the instant invention.

In yet another preferred embodiment, nucleic acid catalysts having chemical 15 modifications which maintain or enhance enzymatic activity is provided. Such a nucleic acid is also, generally, more resistant to nucleases than the corresponding unmodified nucleic acid. Thus, in a cell and/or *in vivo* the activity may not be significantly lowered. As exemplified herein such ribozymes are useful in a cell and/or *in vivo* even if activity over all is reduced 10 fold (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Such 20 modifications herein are said to "maintain" the enzymatic activity on all RNA ribozyme.

In a preferred embodiment, the nucleic acid catalysts of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to smooth muscle cells. The RNA or RNA complexes can be locally administered to relevant tissues through the use of a catheter, infusion pump or stent, with 25 or without their incorporation in biopolymers. Using the methods described herein, other nucleic acid catalysts that cleave target nucleic acid may be derived and used as described above. Specific examples of nucleic acid catalysts of the instant invention are provided below in the Tables and figures.

Sullivan, *et al.*, WO 94/02595, describes the general methods for delivery of nucleic acid catalysts. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, 5 cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination is locally delivered by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or 10 joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme delivery and administration are provided in Sullivan *et al.*, *supra* and Draper *et al.*, WO 93/23569 which have been incorporated by reference herein.

Such nucleic acid catalysts can be delivered exogenously to specific cells as 15 required. In the preferred hammerhead motif the small size (less than 60 nucleotides, preferably between 30-40 nucleotides in length) of the molecule allows the cost of treatment to be reduced.

Therapeutic ribozymes delivered exogenously must remain stable within cells until translation of the target RNA has been inhibited long enough to reduce the levels of 20 the undesirable protein. This period of time varies between hours to days depending upon the disease state. Clearly, ribozymes must be resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of RNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; incorporated by reference herein) have expanded the ability to modify ribozymes by introducing nucleotide modifications to 25 enhance their nuclease stability as described above.

Synthesis, Deprotection, and Purification of Nucleic Acid Catalysts:

Generally, RNA molecules are chemically synthesized and purified by methodologies based on the use of tetrazole to activate the RNA phosphoramidite, ethanolic-NH<sub>4</sub>OH to remove the exocyclic amino protecting groups, tetra-*n*-butylammonium fluoride (TBAF) to remove the 2'-OH alkylsilyl protecting groups, and gel purification and analysis of the deprotected RNA. Examples of chemical synthesis, deprotection, purification and analysis procedures for RNA are provided by Usman et al., 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe et al. *Nucleic Acids Res.* 1990, 18, 5433-5341; Perreault et al. *Biochemistry* 1991, 30 4020-4025; Slim and Gait *Nucleic Acids Res.* 1991, 19, 1183-1188. All the above noted references are all hereby incorporated by reference herein.

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs (e.g., antisense oligonucleotides, hammerhead or the hairpin ribozymes) are used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of the mRNA structure. However, these nucleic acid molecules can also be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985 *Science* 229, 345; McGarry and Lindquist, 1986 *Proc. Natl. Acad. Sci. USA* 83, 399; SullengerScanlon et al., 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet et al., 1992 *Antisense Res. Dev.*, 2, 3-15; Dropulic et al., 1992 *J. Virol.* 66, 1432-41; Weerasinghe et al., 1991 *J. Virol.*, 65, 5531-4; Ojwang et al., 1992 *Proc. Natl. Acad. Sci. USA* 89, 10802-6; Chen et al., 1992 *Nucleic Acids Res.*, 20, 4581-9; Sarver et al., 1990 *Science* 247, 1222-1225; Thompson et al., 1995 *Nucleic Acids Res.* 23, 2259). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a ribozyme (Draper et al., PCT WO93/23569, and Sullivan et al., PCT WO94/02595, both hereby incorporated in their totality by reference herein; Ohkawa et al., 1992 *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira et al., 1991, *Nucleic Acids Res.*, 19,

5125-30; Ventura *et al.*, 1993 *Nucleic Acids Res.*, 21, 3249-55; Chowrira *et al.*, 1994 *J. Biol. Chem.* 269, 25856).

The ribozymes were chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684 and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. Small scale synthesis were conducted on a 394 Applied Biosystems, Inc. synthesizer using a modified 2.5  $\mu$ mol scale protocol with a 5 min coupling step for alkylsilyl protected nucleotides and 2.5 min coupling step for 2'-O-methylated nucleotides. Table II outlines the amounts, and the contact times, of the reagents used in the synthesis cycle. A 6.5-fold excess (163  $\mu$ L of 0.1 M = 16.3  $\mu$ mol) of phosphoramidite and a 24-fold excess of *S*-ethyl tetrazole (238  $\mu$ L of 0.25 M = 59.5  $\mu$ mol) relative to polymer-bound 5'-hydroxyl was used in each coupling cycle. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, were 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer:detritylation solution was 2% TCA in methylene chloride (ABI); capping was performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM I<sub>2</sub>, 49 mM pyridine, 9% water in THF (Millipore). B & J Synthesis Grade acetonitrile was used directly from the reagent bottle. *S*-Ethyl tetrazole solution (0.25 M in acetonitrile) was made up from the solid obtained from American International Chemical, Inc.

Deprotection of the RNA was performed as follows. The polymer-bound oligoribonucleotide, trityl-off, was transferred from the synthesis column to a 4mL glass screw top vial and suspended in a solution of methylamine (MA) at 65 °C for 10 min. After cooling to -20 °C, the supernatant was removed from the polymer support. The support was washed three times with 1.0 mL of EtOH:MeCN:H<sub>2</sub>O/3:1:1, vortexed and

the supernatant was then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, were dried to a white powder.

The base-deprotected oligoribonucleotide was resuspended in anhydrous TEA•HF/NMP solution (250  $\mu$ L of a solution of 1.5mL *N*-methylpyrrolidinone, 750  $\mu$ L 5 TEA and 1.0 mL TEA•3HF to provide a 1.4M HF concentration) and heated to 65°C for 1.5 h. The resulting, fully deprotected, oligomer was quenched with 50 mM TEAB (9 mL) prior to anion exchange desalting.

For anion exchange desalting of the deprotected oligomer, the TEAB solution was loaded onto a Qiagen 500® anion exchange cartridge (Qiagen Inc.) that was prewashed 10 with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA was eluted with 2 M TEAB (10 mL) and dried down to a white powder.

#### Deprotection of RNA:

For high throughput chemical synthesis of oligoribonucleotides, it is important that the two main steps involved in the deprotection of oligoribonucleotides (i.e., aqueous 15 basic treatment to remove exocyclic amino protecting groups and phosphate protecting groups and fluoride treatment to remove the 2'-*OH* alkylsilyl protecting groups such as the tButylDiMethylSilyl) are condensed.

Stinchcomb *et al.*, *supra* describe a time-efficient (~ 2 hrs) one-pot deprotection protocol based on anhydrous methylamine and triethylamine trihydrogen fluoride. Since 20 it has recently been reported that water contamination during fluoride treatment may be detrimental to the efficiency of the desilylation reaction (Hogrefe *et al.*, Nucleic Acids Res. (1993), 21 4739-4741), it is necessary to use an anhydrous solution of base such as a 33% methylamine in absolute ethanol followed by neat triethylamine trihydrofluoride to effectively deprotect oligoribonucleotides in a one-pot fashion. However it may be 25 cumbersome to apply such a protocol to plate format deprotection where the solid-support is preferentially separated from the partially deprotected oligoribonucleotides prior to the 2'-hydroxyl deprotection. Indeed, because the methylamine solution used is

anhydrous, it may not be suitable to solubilize the negatively charged oligoribonucleotides obtained after basic treatment. Therefore, applicant investigated a 1:1 mixture of the ethanolic methylamine solution and different polar additives such as 5 dimethylsulfoxide (DMSO), N,N-dimethylformamide (DMF), methanol, hexamethylphosphoramide (HMPA), 1-methyl-2-pyrrolidinone (NMP) or 2-methoxyethyl ether (glyme). Of all these additives, dimethylsulfoxide is capable of efficiently solubilizing partially deprotected oligoribonucleotides (figure 34). A comparison of the one pot and two pot deprotection methods are outlined and demonstrated in figure 33.

10 The deprotection process commonly involves the deprotection of the exocyclic amino protecting groups by NH<sub>4</sub>OH, which is time consuming (6-24 h) and inefficient. This step is then followed by treatment with TBAF to facilitate the removal of alkylsilyl protecting groups, which again is time consuming and not very effective in achieving efficient deprotection.

15 A recent modification of this two-step strategy for oligoribonucleotide deprotection has been reported by Wincott *et al.*, (*Nucleic Acids Res.*, 1995, 23, 2677-2784) and by Vinayak *et al.*, (*Nucleic Acids Symposium series*, 1995, 33, 123-125). The optimized conditions make use of aqueous methylamine at 65°C for 15 minutes in place of the ammonium hydroxide cocktail to remove exocyclic amino protecting groups while 20 the desilylation treatment needed to remove the 2'-OH alkylsilyl protecting groups utilizes a mixture of triethylamine trihydrogen fluoride (TEA.3HF), N-methyl-pyrrolidinone and triethylamine at 65°C for 90 minutes, thereby replacing tetrabutyl ammonium fluoride.

25 Stinchcomb *et al.*, International PCT Publication No. WO 95/23225 describe a process for one pot deprotection of RNA. On page 73, it states that:

"In an attempt to minimize the time required for deprotection and to simplify the process of deprotection of RNA synthesized on a large scale, applicant describes a one pot

deprotection protocol... According to this protocol, anhydrous methylamine is used in place of aqueous methyl amine. Base deprotection is carried out at 65 °C for 15 minutes and the reaction is allowed to cool for 10 min. Deprotection of 2'-hydroxyl groups is then carried out in the same container for 90 minutes in a TEA•3HF reagent. The reaction is 5 quenched with 16 mM TEAB solution."

This invention concerns a one-pot process for the deprotection of RNA molecules. This invention features a novel method for the removal of protecting groups from the nucleic acid base and 2'-OH groups, which accelerates the process for generating synthetic RNA in a high throughput manner (e.g., in a 96 well format).

10 Chemical synthesis of RNA is generally accomplished using a traditional column format on a RNA synthesizer where only one oligoribonucleotide is synthesized at a time. Simultaneous synthesis of more than one RNA molecule in a time efficient manner requires alternate methods to the traditional column format, such as synthesis in a 96 well plate format where up to 96 RNA molecules can be synthesized at the same time. To 15 expedite this process of simultaneous synthesis of multiple RNA molecules, it is important to accelerate some of the time consuming processes such as the deprotection of RNA following synthesis (*i.e.*, removal of base protecting group, such as the exocyclic amino protecting group and the phosphate protecting groups and the removal of 2'-OH protecting groups, such as the tButylDiMethylSilyl). In a preferred embodiment, the 20 invention features a one-pot process for rapid deprotection of RNA.

Stinchcomb *et al.*, *supra* described a one-pot protocol for RNA deprotection using anhydrous methylamine and triethylamine trihydrogen fluoride. This procedure involves the use of an anhydrous solution of base such as a 33% methylamine in absolute ethanol followed by neat triethylamine trihydrofluoride to effectively deprotect 25 oligoribonucleotides in a one-pot fashion. However such a protocol may be cumbersome for deprotection of RNA synthesized on a plate format, such as a 96 well plate, because it may be necessary to separate the solid-support from the partially deprotected RNA prior to the 2'-hydroxyl deprotection. Also, since the methylamine solution used is anhydrous,

it may be difficult to solubilize the negatively charged oligoribonucleotides obtained after basic treatment. So, in a first aspect the invention features the use of a 1:1 mixture of the ethanolic methylamine solution and a polar additive, such as dimethylsulfoxide (DMSO), N,N-dimethylformamide (DMF), methanol, hexamethylphosphoramide (HMPA), 1-methyl-2-pyrrolidinone (NMP), 2-methoxyethyl ether (glyme) or the like. More specifically, dimethylsulfoxide is used to partially deprotect oligoribonucleotides (Figure 32). A comparison of the one pot and two pot deprotection methods are outlined and demonstrated in Figure 33.

This invention also concerns a rapid (high through-put) deprotection of RNA in a 10 96-well plate format. More specifically rapid deprotection of enzymatic RNA molecules in greater than microgram quantities with high biological activity is featured. It has been determined that the recovery of enzymatically active RNA in high yield and quantity is dependent upon certain critical steps used during its deprotection.

In a preferred embodiment, the invention features a process for one-pot 15 deprotection of RNA molecules comprising protecting groups, comprising the steps of: a) contacting the RNA with a mixture of anhydrous alkylamine (where alkyl can be branched or unbranched, ethyl, propyl or butyl and is preferably methyl, e.g., methylamine), trialkylamine (where alkyl can be branched or unbranched, methyl, propyl or butyl and is preferably ethyl, e.g., ethylamine) and dimethylsulfoxide, preferably in a 20 10:3:13, or 1:0.3:1 proportion at temperature 20-30 °C for about 30-100 minutes, preferably 90 minutes, to remove the exocyclic amino (base) protecting groups and the phosphate protecting group (e.g., 2-cyanoethyl) (vs 4-20 h at 55-65 °C using NH<sub>4</sub>OH/EtOH or NH<sub>3</sub>/EtOH, or 10-15 min at 65°C using 40% aqueous methylamine) under conditions suitable for partial deprotection of the RNA; b) contacting the partially 25 deprotected RNA with anhydrous triethylamine-hydrogen fluoride (3HF•TEA) and heating at about 50-70 °C, preferably at 65 °C, for about 5-30 min, preferably 15 min to remove the 2'-hydroxyl protecting group (vs 8 - 24 h using TBAF, or TEA•3HF for 24 h (Gasparutto *et al. Nucleic Acids Res.* 1992, 20, 5159-5166) (Other alkylamine•HF

complexes may also be used, *e.g.*, trimethylamine or diisopropylethylamine) under conditions suitable for the complete deprotection of the RNA. The reaction can then be quenched by using aqueous ammonium bicarbonate (1.4 M). Although some other buffers can be used to quench the desilylation reaction (*i.e.*, triethylammonium bicarbonate, ammonium acetate), the ammonium bicarbonate buffer is perfectly suited to retain the 5'-*O*-dimethoxytrityl group at the 5'-end of the oligoribonucleotide thereby facilitating a reverse phase-based solid-phase extraction purification protocol.

By "one-pot" deprotection is meant that the process of deprotection RNA is carried out in one container instead of multiple containers as in two-pot deprotection.

10 In another preferred embodiment, the invention features a process for one pot deprotection of RNA molecules comprising protecting groups, comprising the steps of: a) contacting the RNA with a mixture of anhydrous alkylamine (where alkyl can be branched or unbranched, ethyl, propyl or butyl and is preferably methyl, *e.g.*, methylamine), and dimethylsulfoxide, preferably in a 1:1 proportion at 20-30 °C 15 temperature for about 30-100 minutes, preferably 90 minutes, to remove the exocyclic amino (base) protecting groups and the phosphate protecting group (*e.g.*, 2-cyanoethyl) (*vs* 4-20 h at 55-65 °C using NH<sub>4</sub>OH/EtOH or NH<sub>3</sub>/EtOH, or 10-15 min at 65°C using 40% aqueous methylamine) under conditions suitable for partial deprotection of the RNA; b) contacting the partially deprotected RNA with anhydrous 20 triethylamine-hydrogen fluoride (3HF•TEA) and heating at about 50-70 °C, preferably at 65 °C, for about 5-30 min, preferably 15 min to remove the 2'-hydroxyl protecting group (Other alkylamine-HF complexes may also be used, *e.g.*, trimethylamine or diisopropylethylamine) under conditions suitable for the complete deprotection of the RNA. The reaction can then be quenched by using aqueous ammonium bicarbonate (1.4 25 M). Although some other buffers can be used to quench the desilylation reaction (*i.e.*, triethylammonium bicarbonate, ammonium acetate), the ammonium bicarbonate buffer is perfectly suited to retain the 5'-*O*-dimethoxytrityl group at the 5'-end of the

oligoribonucleotide thereby facilitating a reverse phase-based solid-phase extraction purification protocol.

In another aspect the invention features a process for RNA deprotection where the exocyclic amino and phosphate deprotection reaction is performed with the ethanolic 5 methylamine solution at room temperature for about 90 min or at 65°C for 15 min or at 45°C for 30 min or at 35°C for 60 min.

In a preferred embodiment, the process for deprotection of RNA of the present invention is used to deprotect a ribozyme synthesized using a column format as described in (Scaringe *et al.*, *supra*; Wicott *et al.*, *supra*).

10 Inactive hammerhead ribozymes were synthesized by substituting a U for G5 and a U for A14 (numbering from Hertel, K. J., *et al.*, 1992, *Nucleic Acids Res.*, 20, 3252).

The average stepwise coupling yields were >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684).

15 Hairpin ribozymes are synthesized in two parts and annealed to reconstruct the active ribozyme (Chowrima and Burke, 1992 *Nucleic Acids Res.*, 20, 2835-2840). Ribozymes are also synthesized from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51).

20 Ribozymes are modified to enhance stability and/or enhance catalytic activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-flouro, 2'-O-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34; Usman *et al.*, 1994 *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996 *Biochemistry* 6, 14090).

Ribozymes were purified by gel electrophoresis using general methods or are purified by high pressure liquid chromatography (HPLC; See Stinchcomb *et al.*,

International PCT Publication No. WO 95/23225, the totality of which is hereby incorporated herein by reference) and are resuspended in water.

The sequences of the ribozymes that are chemically synthesized, useful in this study, are shown in Tables XII-XIX. Those in the art will recognize that these sequences 5 are representative only of many more such sequences where the enzymatic portion of the ribozyme (all but the binding arms) is altered to affect activity. For example, stem-loop II sequence of hammerhead ribozymes can be altered (substitution, deletion, and/or insertion) to contain any sequences provided a minimum of two base-paired stem structure can form. Similarly, stem-loop IV sequence of hairpin ribozymes, can be 10 altered (substitution, deletion, and/or insertion) to contain any sequence, provided a minimum of two base-paired stem structure can form. Preferably, no more than 200 bases are inserted at these locations. The sequences listed in Tables XII-XIX may be formed of ribonucleotides or other nucleotides or non-nucleotides. Such ribozymes 15 (which have enzymatic activity) are equivalent to the ribozymes described specifically in the Tables.

Nucleotide Triphosphates:

The use of modified nucleotide triphosphates would greatly assist in the combinatorial chemistry. The synthesis of nucleoside triphosphates and their incorporation into nucleic acids using polymerase enzymes has greatly assisted in the 20 advancement of nucleic acid research. The polymerase enzyme utilizes nucleoside triphosphates as precursor molecules to assemble oligonucleotides. Each nucleotide is attached by a phosphodiester bond formed through nucleophilic attack by the 3' hydroxyl group of the oligonucleotide's last nucleotide onto the 5' triphosphate of the next nucleotide. Nucleotides are incorporated one at a time into the oligonucleotide in a 5' to 25 3' direction. This process allows RNA to be produced and amplified from virtually any DNA or RNA templates.

Most natural polymerase enzymes incorporate standard nucleoside triphosphates into nucleic acid. For example, a DNA polymerase incorporates dATP, dTTP, dCTP, and

dGTP into DNA and an RNA polymerase generally incorporates ATP, CTP, UTP, and GTP into RNA. There are however, certain polymerases that are capable of incorporating non-standard nucleoside triphosphates into nucleic acids (Joyce, 1997, *PNAS* 94, 1619-1622, Huang et al., *Biochemistry* 36, 8231-8242).

5 Before nucleosides can be incorporated into RNA transcripts using polymerase enzymes they must first be converted into nucleoside triphosphates which can be recognized by these enzymes. Phosphorylation of unblocked nucleosides by treatment with  $\text{POCl}_3$  and trialkyl phosphates was shown to yield nucleoside 5'-phosphorodichloridates (Yoshikawa et al., 1969, *Bull. Chem. Soc. (Japan)* 42, 3505).

10 Adenosine or 2'-deoxyadenosine 5'-triphosphate was synthesized by adding an additional step consisting of treatment with excess tri-n-butylammonium pyrophosphate in DMF followed by hydrolysis (Ludwig, 1981, *Acta Biochim. et Biophys. Acad. Sci. Hung.* 16, 131-133).

15 Non-standard nucleoside triphosphates are not readily incorporated into RNA transcripts by traditional RNA polymerases. Mutations have been introduced into RNA polymerase to facilitate incorporation of deoxyribonucleotides into RNA (Sousa & Padilla, 1995, *EMBO J.* 14, 4609-4621, Bonner et al., 1992, *EMBO J.* 11, 3767-3775, Bonner et al., 1994, *J. Biol. Chem.* 269, 25120-25128, Aurup et al., 1992, *Biochemistry* 31, 9636-9641).

20 McGee et al., International PCT Publication No. WO 95/35102, describes the incorporation of 2'-NH<sub>2</sub>-NTP's, 2'-F-NTP's, and 2'-deoxy-2'-benzyloxyamino UTP into RNA using bacteriophage T7 polymerase.

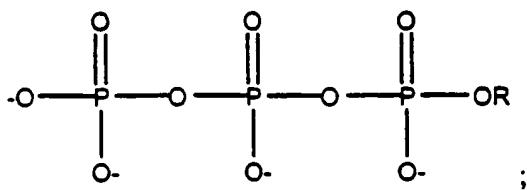
25 Wieczorek et al., 1994, *Bioorganic & Medicinal Chemistry Letters* 4, 987-994, describes the incorporation of 7-deaza-adenosine triphosphate into an RNA transcript using bacteriophage T7 RNA polymerase.

Lin et al., 1994, *Nucleic Acids Research* 22, 5229-5234, reports the incorporation of 2'-NH<sub>2</sub>-CTP and 2'-NH<sub>2</sub>-UTP into RNA using bacteriophage T7 RNA polymerase and

polyethylene glycol containing buffer. The article describes the use of the polymerase synthesized RNA for *in vitro* selection of aptamers to human neutrophil elastase (HNE).

The invention features NTP's having the formula triphosphate-OR, for example the following formula I:

5



where R is any nucleoside; specifically the nucleosides 2'-*O*-methyl-2,6-diaminopurine riboside; 2'-deoxy-2'amino-2,6-diaminopurine riboside; 2'-(*N*-alanyl) amino-2'-deoxy-uridine; 2'-(*N*-phenylalanyl)amino-2'-deoxy-uridine; 2'-deoxy -2'-(*N*- $\beta$ -alanyl) amino ; 10 2'-deoxy-2'-(lysyl) amino uridine; 2'-C-allyl uridine; 2'-*O*-amino-uridine; 2'-*O*-methylthiomethyl adenosine; 2'-*O*-methylthiomethyl cytidine ; 2'-*O*-methylthiomethyl guanosine; 2'-*O*-methylthiomethyl-uridine; 2'-Deoxy-2'-(*N*-histidyl) amino uridine; 2'-deoxy-2'-amino-5-methyl cytidine; 2'-(*N*- $\beta$ -carboxamidine- $\beta$ -alanyl)amino-2'-deoxy-uridine; 2'-deoxy-2'-(*N*- $\beta$ -alanyl)-guanosine; and 2'-*O*-amino-adenosine.

In a second aspect, the invention features a process for the synthesis of pyrimidine nucleotide triphosphate (such as UTP, 2'-*O*-MTM-UTP, dUTP and the like) including the steps of monophosphorylation where the pyrimidine nucleoside is contacted with a mixture having a phosphorylating agent (such as phosphorus oxychloride, phospho-tris-triazolides, phospho-tris-triimidazolides and the like), trialkyl phosphate (such as triethylphosphate or trimethylphosphate or the like) and dimethylaminopyridine (DMAP) 15 under conditions suitable for the formation of pyrimidine monophosphate; and pyrophosphorylation where the pyrimidine monophosphate is contacted with a pyrophosphorylating reagent (such as tributylammonium pyrophosphate) under 20 conditions suitable for the formation of pyrimidine triphosphates.

By "pyrimidines" is meant nucleotides comprising modified or unmodified derivatives of a six membered pyrimidine ring. An example of a pyrimidine is modified or unmodified uridine.

By "nucleotide triphosphate" or "NTP" is meant a nucleoside bound to three inorganic phosphate groups at the 5' hydroxyl group of the modified or unmodified ribose or deoxyribose sugar where the 1' position of the sugar may comprise a nucleic acid base or hydrogen. The triphosphate portion may be modified to include chemical moieties which do not destroy the functionality of the group (*i.e.*, allow incorporation into an RNA molecule).

10 In another preferred embodiment, nucleoside triphosphates (NTP's) of the instant invention are incorporated into an oligonucleotide using an RNA polymerase enzyme. RNA polymerases include but are not limited to mutated and wild type versions of bacteriophage T7, SP6, or T3 RNA polymerases.

15 In yet another preferred embodiment, the invention features a process for incorporating modified NTP's into an oligonucleotide including the step of incubating a mixture having a DNA template, RNA polymerase, NTP, and an enhancer of modified NTP incorporation under conditions suitable for the incorporation of the modified NTP into the oligonucleotide.

20 By "enhancer of modified NTP incorporation" is meant a reagent which facilitates the incorporation of modified nucleotides into a nucleic acid transcript by an RNA polymerase. Such reagents include but are not limited to methanol; LiCl; polyethylene glycol (PEG); diethyl ether; propanol; methyl amine; ethanol and the like.

25 In another preferred embodiment, the modified nucleoside triphosphates can be incorporated by transcription into a nucleic acid molecules including enzymatic nucleic acid, antisense, 2-5A antisense chimera, oligonucleotides, triplex forming oligonucleotide (TFO), aptamers and the like (Stull *et al.*, 1995 *Pharmaceutical Res.* 12, 465).

By "antisense" it is meant a non-nucleic acid catalyst that binds to target RNA by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm *et al.*, 1993 *Nature* 365, 566) interactions and alters the activity of the target RNA (for a review see Stein and Cheng, 1993 *Science* 261, 1004; Agrawal *et al.*, U.S. Patent No. 5,591,721; 5 Agrawal, U.S. Patent No. 5,652,356).

By "2-5A antisense chimera" it is meant, an antisense oligonucleotide containing a 5' phosphorylated 2'-5'-linked adenylate residues. These chimeras bind to target RNA in a sequence-specific manner and activate a cellular 2-5A-dependent ribonuclease which, in turn, cleaves the target RNA (Torrence *et al.*, 1993 *Proc. Natl. Acad. Sci. USA* 90, 1300).

10 By "triplex forming oligonucleotides (TFO)" it is meant an oligonucleotide that can bind to a double-stranded DNA in a sequence-specific manner to form a triple-strand helix. Formation of such triple helix structure has been shown to inhibit transcription of the targeted gene (Duval-Valentin *et al.*, 1992 *Proc. Natl. Acad. Sci. USA* 89, 504).

15 By "oligonucleotide" as used herein is meant a molecule having two or more nucleotides. The polynucleotide can be single, double or multiple stranded and may have modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

20 In yet another preferred embodiment, the modified nucleoside triphosphates of the instant invention can be used for combinatorial chemistry or *in vitro* selection of nucleic acid molecules with novel function. Modified oligonucleotides can be enzymatically synthesized to generate libraries for screening.

25 Nucleoside modifications of bases and sugars, have been discovered in a variety of naturally occurring RNA (e.g., tRNA, mRNA, rRNA; reviewed by Hall, 1971 *The Modified Nucleosides in Nucleic Acids*, Columbia University Press, New York; Limbach *et al.*, 1994 *Nucleic Acids Res.* 22, 2183). In an attempt to understand the biological significance, structural and thermodynamic properties, and nuclease resistance of these nucleoside modifications in nucleic acids, several investigators have chemically

synthesized nucleosides, nucleotides and phosphoramidites with various base and sugar modifications and incorporated them into oligonucleotides.

Uhlmann and Peyman, 1990, *Chem. Reviews* 90, 543, review the use of certain nucleoside modifications to stabilize antisense oligonucleotides.

5 Usman *et al.*, International PCT Publication Nos. WO/93/15187; and WO 95/13378; describe the use of sugar, base and backbone modifications to enhance the nuclease stability of nucleic acid catalysts.

10 Eckstein *et al.*, International PCT Publication No. WO 92/07065 describe the use of sugar, base and backbone modifications to enhance the nuclease stability of nucleic acid catalysts.

Grasby *et al.*, 1994, *Proc. Indian Acad. Sci.*, 106, 1003, review the "applications of synthetic oligoribonucleotide analogues in studies of RNA structure and function".

Eaton and Pieken, 1995, *Annu. Rev. Biochem.*, 64, 837, review sugar, base and backbone modifications that enhance the nuclease stability of RNA molecules.

15 Rosemeyer *et al.*, 1991, *Helvetica Chem. Acta*, 74, 748, describe the synthesis of 1-(2'-deoxy- $\beta$ -D-xylofuranosyl) thymine-containing oligodeoxynucleotides.

Seela *et al.*, 1994, *Helvetica Chem. Acta*, 77, 883, describe the synthesis of 1-(2'-deoxy- $\beta$ -D-xylofuranosyl) cytosine-containing oligodeoxynucleotides.

20 Seela *et al.*, 1996, *Helvetica Chem. Acta*, 79, 1451, describe the synthesis xylose-DNA containing the four natural bases.

In another preferred embodiment, catalytic activity of the molecules described in the instant invention can be optimized as described by Draper *et al.*, *supra*. The details will not be repeated here, but include altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications (base, sugar and/or phosphate) that

prevent their degradation by serum ribonucleases and/or enhance their enzymatic activity (see e.g., Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken *et al.*, 1991 *Science* 253, 314; Usman and Cedergren, 1992 *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. 5 WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al.*, *supra*; all of these describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of enzymatic RNA molecules). Modifications which enhance their efficacy in cells, and removal of bases from stem loop structures to shorten RNA synthesis times and reduce chemical requirements are desired. (All these publications are hereby incorporated by reference herein.).

By "enhanced enzymatic activity" is meant to include activity measured in cells and/or *in vivo* where the activity is a reflection of both catalytic activity and ribozyme stability. In this invention, the product of these properties is increased or not significantly 15 (less than 10 fold) decreased *in vivo* compared to an all RNA ribozyme.

In yet another preferred embodiment, nucleic acid catalysts having chemical modifications which maintain or enhance enzymatic activity is provided. Such nucleic acid is also generally more resistant to nucleases than unmodified nucleic acid. Thus, in a cell and/or *in vivo* the activity may not be significantly lowered. As exemplified herein 20 such ribozymes are useful in a cell and/or *in vivo* even if activity over all is reduced 10 fold (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Such ribozymes herein are said to "maintain" the enzymatic activity on all RNA ribozyme.

In a most preferred embodiment the invention features a method of synthesizing 25 ribozyme libraries of various sizes. This invention describes methods to chemically synthesize ribozyme libraries of various sizes from suitable nucleoside analogs.

*Considerations for the selection of nucleotide building blocks and determination of coupling efficiency:* In addition to structural considerations (hydrogen bond donors and

acceptors, stacking properties, pucker orientation of sugars, hydrophobicity or hydrophilicity of some subgroups constitutive of the nucleotides) that may lead to the selection of a specific nucleotide to be included in the design of a ribozyme library, one of the important features that needs to be considered when selecting nucleotide building blocks is the chemical compatibility of such building blocks with ribozyme synthesis. A "nucleotide building block" is a nucleoside or nucleoside analog that possess a suitably protected phosphorus atom at the oxidation state V reacting readily, upon activation, to give a P<sup>V</sup>-containing internucleoside linkage. A suitable nucleoside building block may also contain a phosphorus atom at the oxidation state III reacting readily, upon activation, to give a P<sup>III</sup>-containing internucleoside linkage that can be oxidized to the desired P<sup>V</sup>-containing internucleoside linkage. Applicant has found that the phosphoramidite chemistry (P<sup>III</sup>) is a preferred coupling method for ribozyme library synthesis. There are several other considerations while designing and synthesizing certain ribozyme libraries, such as: a) the coupling efficiencies of the nucleotide building blocks considered for a ribozyme library should not fall below 90% to provide a majority of full-length ribozyme; b) the nucleotide building blocks should be chemically stable to the selected synthesis and deprotection conditions of the particular ribozyme library; c) the deprotection schemes for the nucleotide building blocks incorporated into a ribozyme library, should be relatively similar and be fully compatible with ribozyme deprotection protocols. In particular, nucleoside building blocks requiring extended deprotection or that cannot sustain harsh treatment should be avoided in the synthesis of a ribozyme library. Typically, the reactivity of the nucleotide building blocks should be optimum when diluted to 100 mM to 200 mM in non-protic and relatively polar solvent. Also the deprotection condition using 3:1 mixture of ethanol and concentrated aqueous ammonia at 65 degrees C. for 4 hours followed by a fluoride treatment as exemplified in Wincott *et al. supra*, is particularly useful for ribozyme synthesis and is a preferred deprotection pathway for such nucleotide building blocks.

In one preferred embodiment, a "nucleotide building block mixing" approach to generate ribozyme libraries is described. This method involves mixing various nucleotide

building blocks together in proportions necessary to ensure equal representation of each of the nucleotide building blocks in the mixture. This mixture is incorporated into the ribozyme at position(s) selected for randomization.

The nucleotide building blocks selected for incorporation into a ribozyme library, 5 are typically mixed together in appropriate concentrations, in reagents, such as anhydrous acetonitrile, to form a mixture with a desired phosphoramidite concentration. This approach for combinatorial synthesis of a ribozyme library with one or more random positions within the ribozyme (X as described above) is particularly useful since a standard DNA synthesizer can handle a building block mixture similar to a building block 10 solution containing a single building block. Such a nucleotide building block mixture is coupled to a solid support or to a growing ribozyme sequence attached to a solid-support. To ensure that the ribozyme library synthesized achieves the desired complexity, the scale 15 of the synthesis is increased substantially above that of the total complexity of the library. For example, a 2.5  $\mu$ mole ribozyme synthesis provides  $\sim 3 \times 10^{17}$  ribozyme molecules corresponding to sub-nanomolar amounts of each member of a billion compounds ribozyme library.

Divinylbenzene highly cross-linked polystyrene solid-support constitutes the preferred stationary phase for ribozyme library synthesis. However, other solid-support 20 systems utilized in DNA or RNA synthesis can also be used for ribozyme library synthesis. This includes silica-based solid-supports such as controlled-pore glass (CPG) or polymeric solid-supports such as all types of derivatized polystyrene resins, grafted polymers of chloromethylated polystyrene crosslinked with ethylene glycol, oligoethylene glycol.

Because of different coupling kinetics of the nucleotide building blocks present in 25 a mixture, it is necessary to evaluate the relative incorporation of each of the members of the mixture and to adjust, if needed, the relative concentration of the building blocks in the mixture to get equimolar representation, compensating thereby the kinetic parameter. Typically a building block that presents a slow coupling kinetic will be over-represented

in the mixture and vice versa for a building block that presents a fast coupling kinetic. When equimolar incorporation is sought, acceptable limits for unequal incorporation may generally be +/-10%.

Synthesis of a random ribozyme library can be performed either with the mixture 5 of desired nucleotide building blocks, or with a combination of certain random positions (obtained by using one or more building block mixtures) and one or more fixed positions that can be introduced through the incorporation of a single nucleotide building block reagent. For instance, in the oligonucleotide model 5'-TT XXXX TTB-3' used in example 2 *infra*, the positions from 3'-end 1 is fixed as 2'-deoxy-inverted abasic ribose 10 (B), positions 2, 3, 8 and 9 have been fixed as 2'-deoxy-thymidine (T) while the X positions 4-7 correspond to an approximately equimolar distribution of all the nucleotide building blocks that make up the X mixture.

In another preferred embodiment, a "mix and split" approach to generate ribozyme 15 libraries is described. This method is particularly useful when the number of selected nucleotide building blocks to be included in the library is large and diverse (greater than 5 nucleotide building blocks) and/or when the coupling kinetics of the selected nucleotide building blocks do not allow competitive coupling even after relative concentration 20 adjustments and optimization. This method involves a multi-step process wherein the solid support used for ribozyme library synthesis is "split" (divided) into equal portions, (the number of portions is equal to the number of different nucleotide building blocks (n) 25 chosen for incorporation at one or more random positions within the ribozyme). For example, if there are 10 different nucleotide building blocks chosen for incorporation at one or more positions in the ribozyme library, then the solid support is divided into 10 different portions. Each portion is independently coupled to one of the selected nucleotide building blocks followed by mixing of all the portions of solid support. The 30 ribozyme synthesis is then resumed as before the division of the building blocks. This enables the synthesis of a ribozyme library wherein one or more positions within the ribozyme is random. The number of "splitting" and "mixing" steps is dependent on the

number of positions that are random within the ribozyme. For example if three positions are desired to be random then three different splitting and mixing steps are necessary to synthesize the ribozyme library.

Random ribozyme libraries are synthesized using a non-competitive coupling procedure where each of the selected nucleotide analogs "n" separately couple to an inverse "n" ( $1/n$ ) number of aliquots of solid-support or of a growing ribozyme chain on the solid-support. A very convenient way to verify completeness of the coupling reaction is the use of a standard spectrophotometric DMT assay (Oligonucleotide Synthesis, A Practical Approach, ed. M. Gait, pp 48, IRL Press, Oxford, UK; incorporated by reference herein). These aliquots may be subsequently combined, mixed and split into one new aliquot. A similar approach to making oligonucleotide libraries has recently been described by Cook *et al.*, (US Patent No. 5,587,471) and is incorporated by reference herein.

#### Nucleotide Synthesis

Addition of dimethylaminopyridine (DMAP) to the phosphorylation protocols known in the art can greatly increase the yield of nucleoside monophosphates while decreasing the reaction time (Fig. 29). Synthesis of the nucleosides of the invention have been described in several publications and Applicants previous applications (Beigelman *et al.*, International PCT publication No. WO 96/18736; Dudzcy *et al.*, Int. PCT Pub. No. 15 WO 95/11910; Usman *et al.*, Int. PCT Pub. No. WO 95/13378; Matulic-Adamic *et al.*, 20 1997, *Tetrahedron Lett.* 38, 203; Matulic-Adamic *et al.*, 1997, *Tetrahedron Lett.* 38, 1669; all of which are incorporated herein by reference). These nucleosides are dissolved 25 in triethyl phosphate and chilled in an ice bath. Phosphorus oxychloride (POCl<sub>3</sub>) is then added followed by the introduction of DMAP. The reaction is then warmed to room temperature and allowed to proceed for 5 hours. This reaction allows the formation of nucleoside monophosphates which can then be used in the formation of nucleoside triphosphates. Tributylamine is added followed by the addition of anhydrous acetonitrile and tributylammonium pyrophosphate. The reaction is then quenched with TEAB and

stirred overnight at room temperature (about 20C). The triphosphate is purified using column purification and HPLC and the chemical structure is confirmed using NMR analysis. Those skilled in the art will recognize that the reagents, temperatures of the reaction, and purification methods can easily be alternated with substitutes and equivalents and still obtain the desired product.

The invention provides nucleoside triphosphates which can be used for a number of different functions. The nucleoside triphosphates formed from nucleosides found in table III are unique and distinct from other nucleoside triphosphates known in the art. Incorporation of modified nucleotides into DNA or RNA oligonucleotides can alter the properties of the molecule. For example, modified nucleotides can hinder binding of nucleases, thus increasing the chemical half-life of the molecule. This is especially important if the molecule is to be used for cell culture or *in vivo*. It is known in the art that the introduction of modified nucleotides into these molecules can greatly increase the stability and thereby the effectiveness of the molecules (Burgin *et al.*, 1996, *Biochemistry* 35, 14090-14097; Usman *et al.*, 1996, *Curr. Opin. Struct. Biol.* 6, 527-533).

Modified nucleotides are incorporated using either wild type and mutant polymerases. For example, mutant T7 polymerase is used in the presence of modified nucleotide triphosphate(s), DNA template and suitable buffers. Those skilled in the art will recognize that other polymerases and their respective mutant versions can also be utilized for the incorporation of NTP's of the invention. Nucleic acid transcripts were detected by incorporating radiolabelled nucleotides ( $\alpha$ -<sup>32</sup>P NTP). The radiolabeled NTP contained the same base as the modified triphosphate being tested. The effects of methanol, PEG and LiCl were tested by adding these compounds independently or in combination. Detection and quantitation of the nucleic acid transcripts was performed using a Molecular Dynamics PhosphorImager. Efficiency of transcription was assessed by comparing modified nucleotide triphosphate incorporation with all-ribonucleotide incorporation control. Wild type polymerase was used to incorporate NTP's using the manufacturers buffers and instructions (Boehringer Mannheim).

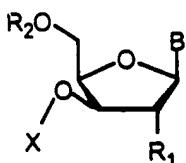
Transcription Conditions

Incorporation rates of modified nucleoside triphosphates into oligonucleotides can be increased by adding to traditional buffer conditions, several different enhancers of modified NTP incorporation. Applicant has utilized methanol and LiCl in an attempt to 5 increase incorporation rates of dNTP using RNA polymerase. These enhancers of modified NTP incorporation can be used in different combinations and ratios to optimize transcription. Optimal reaction conditions differ between nucleoside triphosphates and can readily be determined by standard experimentation. Overall however, inclusion of 10 enhancers of modified NTP incorporation such as methanol or inorganic compound such as lithium chloride, have been shown by the applicant to increase the mean transcription rates.

Administration of Nucleoside mono, di or triphosphates

The nucleotide monophosphates, diphosphates, or triphosphates can be used as a therapeutic agent either independently or in combination with other pharmaceutical components. These molecules of the inventions can be administered to patients using the 15 methods of Sullivan *et al.*, PCT WO 94/02595. Molecules of the invention may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation 20 into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the modified nucleotide triphosphate, diphosphate or monophosphate/vehicle combination is 25 locally delivered by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of delivery and administration are provided in Sullivan *et al.*, supra and Draper *et al.*, PCT WO93/23569 which have been incorporated by reference herein.

This invention further relates to a compound having the Formula II:



wherein, R<sub>1</sub> is OH, O-R<sub>3</sub>, where R<sub>3</sub> is independently a moiety selected from a group consisting of alkyl, alkenyl, alkynyl, aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester; C-R<sub>3</sub>, where R<sub>3</sub> is independently a moiety selected from a group consisting of alkyl, alkenyl, alkynyl, aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester; halo, NHR<sub>4</sub> (R<sub>4</sub>=alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl), or OCH<sub>2</sub>SCH<sub>3</sub> (methylthiomethyl), ONR<sub>5</sub>, where R<sub>5</sub> is independently H, aminoacyl group, peptidyl group, biotinyl group, cholesteryl group, lipoic acid residue, retinoic acid residue, folic acid residue, ascorbic acid residue, nicotinic acid residue, 6-aminopenicillanic acid residue, 7-aminocephalosporanic acid residue, alkyl, alkenyl, alkynyl, aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide or ester, ON=R<sub>6</sub>, where R<sub>6</sub> is independently pyridoxal residue, pyridoxal-5-phosphate residue, 13-cis-retinal residue, 9-cis-retinal residue, alkyl, alkenyl, alkynyl, alkylaryl, carbocyclic alkylaryl, or heterocyclic alkylaryl; B is independently a nucleotide base or its analog or hydrogen; X is independently a phosphorus-containing group; and R<sub>2</sub> is independently blocking group or a phosphorus-containing group.

Specifically, an "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxy, cyano, alkoxy, NO<sub>2</sub> or N(CH<sub>3</sub>)<sub>2</sub>, amino, or SH.

The term "alkenyl" group refers to unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic

groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, NO<sub>2</sub>, halogen, N(CH<sub>3</sub>)<sub>2</sub>, amino, or SH.

5        The term "alkynyl" refers to an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably,  
10      hydroxyl, cyano, alkoxy, =O, =S, NO<sub>2</sub> or N(CH<sub>3</sub>)<sub>2</sub>, amino or SH.

An "aryl" group refers to an aromatic group which has at least one ring having a conjugated p electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) on aryl groups are halogen, trihalomethyl, hydroxyl, SH, cyano, alkoxy, alkyl, alkenyl, alkynyl, 15 and amino groups.

An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above).

"Carbocyclic aryl" groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted.

20        "Heterocyclic aryl" groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted.

An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or  
25      hydrogen.

An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, or alkylaryl.

A "blocking group" is a group which is able to be removed after polynucleotide synthesis and/or which is compatible with solid phase polynucleotide synthesis.

A "phosphorus containing group" can include phosphorus in forms such as 5 dithioates, phosphoramidites and/or as part of an oligonucleotide.

In a preferred embodiment, the invention features a process for synthesis of the compounds of formula II.

In a preferred embodiment the invention features a process for the synthesis of a xylofuranosyl nucleoside phosphoramidite comprising the steps of: a) oxidation of a 2' and 5'-protected ribonucleoside with a an oxidant such as chromium oxide/pyridine/acetic anhydride, dimethylsulfoxide/acetic anhydride, or Dess-Martin reagent (periodinane) followed by reduction with a reducing agent such as, triacetoxy sodium borohydride, sodium borohydride, or lithium borohydride, under conditions suitable for the formation of 2' and 5'-protected xylofuranosyl nucleoside; b) phosphorylation under conditions suitable for the formation of xylofuranosyl nucleoside phosphoramidite.

In yet another preferred embodiment, the invention features the incorporation of the compounds of Formula II into polynucleotides. These compounds can be incorporated into polynucleotides enzymatically. For example by using bacteriophage T7 RNA polymerase, these novel nucleotide analogs can be incorporated into RNA at one or more positions (Milligan *et al.*, 1989, *Methods Enzymol.*, 180, 51). Alternatively, novel nucleoside analogs can be incorporated into polynucleotides using solid phase synthesis (Brown and Brown, 1991, in *Oligonucleotides and Analogues: A Practical Approach*, p. 1, ed. F. Eckstein, Oxford University Press, New York; Wincott *et al.*, 1995, *Nucleic Acids Res.*, 23, 2677; Beaucage & Caruthers, 1996, in *Bioorganic Chemistry: Nucleic Acids*, p 36, ed. S. M. Hecht, Oxford University Press, New York).

The compounds of Formula II can be used for chemical synthesis of nucleotide-tri-phosphates and/or phosphoramidites as building blocks for selective incorporation into oligonucleotides. These oligonucleotides can be used as an antisense molecule, 2'-SA antisense chimera, triplex forming oligonucleotides (TFO) or as an nucleic acid catalyst.

5 The oligonucleotides can also be used as probes or primers for synthesis and/or sequencing of RNA or DNA.

The compounds of the instant invention can be readily converted into nucleotide diphosphate and nucleotide triphosphates using standard protocols (for a review see Hutchinson, 1991, in *Chemistry of Nucleosides and Nucleotides*, v.2, pp 81-160, Ed. L. B.

10 Townsend, Plenum Press, New York, USA; incorporated by reference herein).

The compounds of Formula II can also be independently or in combination used as an antiviral, anticancer or an antitumor agent. These compounds can also be independently or in combination used with other antiviral, anticancer or an antitumor agents.

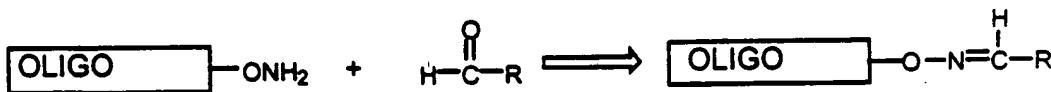
15 In one of the preferred embodiments of the inventions herein, the nucleic acid catalyst is formed in a hammerhead or hairpin motif, but may also be formed in the motif of a hepatitis d virus, group I intron, group II intron or RNaseP RNA (in association with an RNA guide sequence) or *Neurospora* VS RNA. Examples of such hammerhead motifs are described by Dreyfus, *supra*, Rossi *et al.*, 1992, *AIDS Research and Human*  
20 *Retroviruses* 8, 183; of hairpin motifs by Hampel *et al.*, EP0360257, Hampel and Tritz,  
1989 *Biochemistry* 28, 4929, Feldstein *et al.*, 1989, *Gene* 82, 53, Haseloff and Gerlach,  
1989, *Gene*, 82, 43, and Hampel *et al.*, 1990 *Nucleic Acids Res.* 18, 299; of the hepatitis d  
virus motif is described by Perrotta and Been, 1992 *Biochemistry* 31, 16; of the RNaseP  
motif by Guerrier-Takada *et al.*, 1983 *Cell* 35, 849; Forster and Altman, 1990, *Science*  
25 249, 783; Li and Altman, 1996, *Nucleic Acids Res.* 24, 835; *Neurospora* VS RNA  
ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696;  
Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Collins and Olive,  
1993 *Biochemistry* 32, 2795-2799; Guo and Collins, 1995, *EMBO. J.* 14, 363); Group II

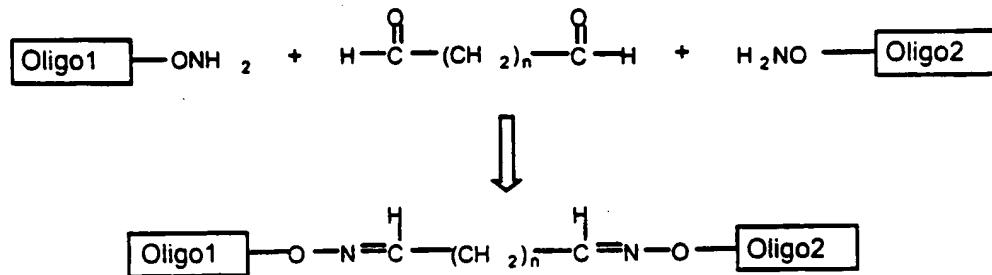
introns are described by Griffin *et al.*, 1995, *Chem. Biol.* 2, 761; Michels and Pyle, 1995, *Biochemistry* 34, 2965; Pyle *et al.*, International PCT Publication No. WO 96/22689; and of the Group I intron by Cech *et al.*, U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in a nucleic acid catalyst of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

In a preferred embodiment, a polynucleotide of the invention would bear one or more 2'-hydroxylamino functionalities attached directly to the monomeric unit or through the use of an appropriate spacer. Since oligonucleotides have neither aldehyde nor hydroxylamino groups, the formation of an oxime would occur selectively using oligo as a polymeric template. This approach would facilitate the attachment of practically any molecule of interest (peptides, polyamines, coenzymes, oligosaccharides, lipids, etc.) directly to the oligonucleotide using either aldehyde or carboxylic function in the molecule of interest.

**Scheme 1. Post synthetic Oxime Bond Formation**

20



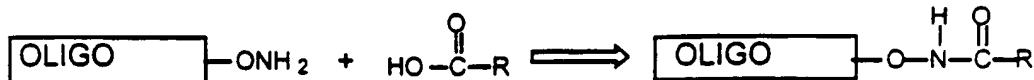
**Scheme 2. Chemical Ligation of Oligonucleotides****5 Advantages of oxime bond formation:**

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- The oximation reaction proceeds in water
- Quantitative yields
- Hydrolytic stability in a wide pH range (5 - 8)
- The amphoteric nature of oximes allows them to act either as weak acids or weak bases.
- Oximes exhibit a great tendency to complex with metal ions

15

In yet another preferred embodiment, the aminoxy "tether" in oligonucleotides, such as a ribozyme, is reacted with different compounds bearing carboxylic groups (e.g., aminoacids, peptides, "cap" structures, etc.) resulting in the formation of oxyamides as shown below.

**Scheme 3. Post synthetic oxyamide bond formation**Target Discovery:

5        Applicant has developed an efficient and rapid method for screening libraries of catalytic nucleic acid molecules capable of performing a desired function in a cell. The invention also features the use of a catalytic nucleic acid library to modulate certain attributes or processes in a biological system, such as a mammalian cell, and to identify and isolate a) nucleic acid catalysts from the library involved in modulating the cellular

10      process/attribute of interest; and b) modulators of the desired cellular process/attribute using the sequence of the nucleic acid catalyst.

More specifically, the method of the instant invention involves designing and constructing a catalytic nucleic acid library, where the catalytic nucleic acid includes a catalytic and a substrate binding domain, and the substrate binding domain (arms) are randomized. This library of catalytic nucleic acid molecules with randomized binding arm(s) are used to modulate certain processes/attributes in a biological system. The method described in this application involves simultaneous screening of a library or pool of catalytic nucleic acid molecules with various substitutions at one or more positions and selecting for ribozymes with desired function or characteristics or attributes. This invention also features a method for constructing and selecting for catalytic nucleic acid molecules for their ability to cleave a given target nucleic acid molecule or an unknown target nucleic acid molecule (e.g., RNA), and to inhibit the biological function of that target molecule or any protein encoded by it.

It is not necessary to know either the sequence or the structure of the target nucleic acid molecule in order to select for catalytic nucleic acid molecules capable of cleaving the target in this cellular system. The cell-based screening protocol described in the

instant invention (*i.e.*, one which takes place inside a cell) offers many advantages over extracellular systems, because the synthesis of large quantities of RNA by enzymatic or chemical methods prior to assessing the efficacy of the catalytic nucleic acid molecules is not necessary. The invention further describes a rapid method of using catalytic nucleic acid molecule libraries to identify the biological function of a gene sequence inside a cell. 5 Applicant describes a method of using catalytic nucleic acid molecule libraries to identify a nucleic acid molecule, such as a gene, involved in a biological process; this nucleic acid molecule may be a known molecule with a known function, or a known molecule with a previously undefined function or an entirely novel molecule. This is a rapid means for 10 identifying, for example, genes involved in a cellular pathway, such as cell proliferation, cell migration, cell death, and others. This method of gene discovery is not only a novel approach to studying a desired biological process but also a means to identify active reagents that can modulate this cellular process in a precise manner.

15 Applicant describes herein, a general approach for simultaneously assaying the ability of one or more members of a catalytic nucleic acid molecule library to modulate certain attributes/process(es) in a biological system, such as plants, animals or bacteria, involving introduction of the library into a desired cell and assaying for changes in a specific "attribute," "characteristic" or "process." The specific attributes may include cell proliferation, cell survival, cell death, cell migration, angiogenesis, tumor volume, tumor 20 metastasis, levels of a specific mRNA(s) in a cell, levels of a specific protein(s) in a cell, levels of a specific protein secreted, cell surface markers, cell morphology, cell differentiation pattern, cartilage degradation, transplantation, restenosis, viral replication, viral load, and the like. By modulating a specific biological pathway using a catalytic nucleic acid molecule library, it is possible to identify the gene(s) involved in that 25 pathway, which may lead to the discovery of novel genes, or genes with novel function. This method provides a powerful tool to study gene function inside a cell. This approach also offers the potential for designing novel catalytic oligonucleotides, identifying ribozyme accessible sites within a target, and for identifying new nucleic acid targets for ribozyme-mediated modulation of gene expression.

In another aspect the invention involves synthesizing a Random Binding Arm Nucleic Acid Catalyst Library (Random Library) and simultaneously testing all members of the Random Library in cells. This library has ribozymes with random substrate binding arm(s) and a defined catalytic domain. Cells with an altered attribute (such as 5 inhibition of cell proliferation) as a result of interaction with the members of the Random Library are selected and the sequences of the ribozymes from these cells are determined. Sequence information from the binding arm(s) of these ribozymes can be used to isolate nucleic acid molecules that are likely to be involved in the pathway responsible for the desired cellular attribute using standard technology known in the art, e.g., nucleic acid 10 amplification using techniques such as polymerase chain reaction (PCR). This method is a powerful means to isolate new genes or genes with new function.

By "Random Library" as used herein is meant ribozyme libraries comprising all possible variants in the binding arm (s) of a given ribozyme motif. Here the complexity and the content of the library is not defined. The Random Library is expected to comprise 15 sequences complementary to every potential target sequence, for the ribozyme motif chosen, in the genome of an organism. This Random Library can be used to screen for ribozyme cleavage sites in a known target sequence or in a unknown target. In the first instance, the Random Library is introduced into the cell of choice and the expression of the known target gene is assayed. Cells with an altered expression of the target will yield 20 the most effective ribozyme against the known target. In the second instance, the Random Library is introduced into the cell of choice and the cells are assayed for a specific attribute, for example, survival of cells. Cells that survive the interaction with the Random Library are isolated and the ribozyme sequence from these cells is determined. The sequence of the binding arm of the ribozyme can then be used as probes to isolate the 25 gene(s) involved in cell death. Because, the ribozyme(s) from the Random Library is able to modulate (e.g., down regulate) the expression of the gene(s) involved in cell death, the cells are able to survive under conditions where they would have otherwise died. This is a novel method of gene discovery. This approach not only provides the information about mediators of certain cellular processes, but also provides a means to modulate the

expression of these modulators. This method can be used to identify modulators of any cell process in any organism, including but not limited to mammals, plants and bacteria.

The invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high degree of specificity for the nucleic acid sequence of a desired target. The nucleic acid catalyst is preferably targeted to a highly conserved sequence region of a target such that specific diagnosis and/or treatment of a disease or condition can be provided with a single enzymatic nucleic acid.

In a first aspect the invention features a method for identifying one or more nucleic acid molecules, such as gene(s), involved in a process (such as, cell growth, proliferation, apoptosis, morphology, angiogenesis, differentiation, migration, viral multiplication, drug resistance, signal transduction, cell cycle regulation, temperature sensitivity, chemical sensitivity and others) in a biological system, such as a cell. The method involves the steps of: a) providing a random library of nucleic acid catalysts, with a substrate binding domain and a catalytic domain, where the substrate binding domain has a random sequence, to the biological system under conditions suitable for the process to be altered; b) identifying any nucleic acid catalyst present in that biological system where the process has been altered by any nucleic acid catalyst; and c) determining the nucleotide sequence of at least a portion of the binding arm of such a nucleic acid catalyst to allow identification of the nucleic acid molecule involved in the process in that biological system.

In a related aspect the invention features a method for identification of a nucleic acid molecule capable of modulating a process in a biological system. The method includes: a) introducing a library of nucleic acid catalysts with a substrate binding domain and a catalytic domain, where the substrate binding domain has a random sequence, into the biological system under conditions suitable for modulating the process; and b) determining the nucleotide sequence of at least a portion of the substrate binding domain of any nucleic acid catalyst from a biological system where the process has been

modulated to allow said identification of the nucleic acid molecule capable of modulating said process in that biological system.

In a second aspect, the invention further concerns a method for identification of a nucleic acid catalyst capable of modulating a process in a biological system. This involves: a) introducing a library of nucleic acid catalysts with a substrate binding domain and a catalytic domain, where the substrate binding domain has a random sequence, into the biological system under conditions suitable for modulating the process; and b) identifying any nucleic acid catalyst from a biological system where the process has been modulated.

By "enzymatic portion" or "catalytic domain" is meant that portion/region of the ribozyme essential for cleavage of a nucleic acid substrate (for example see Figure 3).

By "nucleic acid molecule" as used herein is meant a molecule having nucleotides. The nucleic acid can be single, double or multiple stranded and may comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof. An example of a nucleic acid molecule according to the invention is a gene which encodes for macromolecule such as a protein.

The "biological system" as used herein may be a eukaryotic system or a prokaryotic system, may be a bacterial cell, plant cell or a mammalian cell, or may be of plant origin, mammalian origin, yeast origin, Drosophila origin, or archebacterial origin.

This invention further relates to novel nucleic acid molecules with catalytic activity, which are particularly useful for cleavage of RNA or DNA. The nucleic acid catalysts of the instant invention are distinct from other nucleic acid catalysts known in the art. This invention also relates to a method of screening variants of nucleic acid catalysts using standard nucleotides or modified nucleotides. Applicant has determined an efficient method for screening libraries of catalytic nucleic acid molecules, particularly those with chemical modifications at one or more positions. The method described in this application involves systematic screening of a library or pool of ribozymes with various

substitutions at one or more positions and selecting for ribozymes with desired function or characteristic or attribute.

In one preferred embodiment, a method for identifying a nucleic acid molecule involved in a process in a cell is described, including the steps of: a) synthesizing a library 5 of nucleic acid catalysts, having a substrate binding domain and a catalytic domain, where the substrate binding domain has a random sequence; b) testing the library in the cell under conditions suitable to cause the process in the cell to be altered (such as: inhibition of cell proliferation, inhibition of angiogenesis, modulation of growth and /or differentiation, and others); c) isolating and enriching the cell with the altered process; d) 10 identifying and isolating the nucleic acid catalyst in the altered cell; e) using an oligonucleotide, having the sequence homologous to the sequence of the substrate binding domain of the nucleic acid catalyst isolated from the altered cell, as a probe to isolate the nucleic acid molecule from the cell or the altered cell. Those nucleic acid molecules identified using the selection/screening method described above are likely to be involved 15 in the process that was being assayed for alteration by the member(s) of the ribozyme library. These nucleic acid molecules may be new gene sequences, or known gene sequences, with a novel function. One of the advantages of this method is that nucleic acid sequences, such as genes, involved in a biological process, such as differentiation, cell growth, disease processes including cancer, tumor angiogenesis, arthritis, 20 cardiovascular disease, inflammation, restenosis, vascular disease and the like, can be readily identified using the Random Library approach. Thus theoretically, one Random Library for a given ribozyme motif can be used to assay any process in any biological system.

In another preferred embodiment the invention involves synthesizing a Defined 25 Arm Nucleic Acid Catalyst Library (Defined Library) and simultaneously testing it against known targets in a cell. The library includes ribozymes with binding arm(s) of known complexity (Defined) and a defined catalytic domain. Modulation of expression of the target gene by ribozymes in the library will cause the cells to have an altered

phenotype. Such cells are isolated and the ribozymes in these cells are the ones most suited for modulating the expression of the desired gene in the cell.

By "Defined Library" as used herein is meant a library of nucleic acid catalysts, wherein each member nucleic acid catalyst is designed and produced independently, then 5 added to the library. Thus, the content, complexity (number of different ribozymes contained in the library) and ratios of library members are defined at the outset. Defined Library comprises  $\geq 2$  ribozymes. The process involves screening the sequence of the known target RNA for all possible sites that can be cleaved by a given ribozyme motif and as described, for example in McSwiggen, US Patent No. 5,525,468, incorporated by 10 reference herein. Synthesizing a representative number of different ribozymes against the target sequence. Combining the ribozymes and introducing the pooled ribozymes into a biological system comprising the target RNA under conditions suitable to facilitate modulation of the expression of the target RNA in said biological system.

#### Screening of Nucleic Acid Catalysts

15 Applicant describes herein, a general combinatorial approach for assaying ribozyme variants based on ribozyme activity and/or a specific "attribute" of a ribozyme, such as the cleavage rate, cellular efficacy, stability, delivery, localization and the like. Variations of this approach also offer the potential for designing novel catalytic oligonucleotides, identifying ribozyme accessible sites within a target, and for identifying 20 new nucleic acid targets for ribozyme-mediated modulation of gene expression.

In one preferred embodiment, the method relies upon testing mixtures (libraries) of ribozymes with various nucleotides, nucleotide analogs, or other analog substitutions, rather than individual ribozymes, to rapidly identify the nucleotide, nucleotide analog, or other analog that is variable at one or more positions within a ribozyme. In the first step 25 (step 1, Figure 2), a desired number of positions (for example, 3 positions as shown in Figure 2) are chosen for variation in a first ribozyme motif (Starting Ribozyme); there is no requirement on the number of positions that can be varied and these positions may or

may not be phylogenetically conserved for the ribozyme. In addition, these position may reside within the catalytic core, binding arms, or accessory domains. The number of positions that are chosen to be varied defines the number of "Classes" of ribozyme libraries that will be synthesized. In the example illustrated in Figure 2, three positions 5 (designated positions 1, 2 and 3) are varied, so three different Classes of ribozyme pool are synthesized. In the next step (step 2), ribozyme pools are synthesized containing a random mixture of different nucleotides, nucleotide analogs, or other analogs at all of the desired positions (designated "X") to be varied except one, which is the "fixed" position (designated "F"). The fixed position contains a specific nucleotide, nucleotide analog or other analog. There is no requirement for the number of nucleotides, or analogs be used. The number of nucleotides or analogs defines the number of pools (designated n) in each 10 Class. For example if ten different nucleotides or analogs are chosen, ten different pools (n=10) will be synthesized for each Class; each of the pools will contain a specific modification at one fixed position (designated F) but will contain an equal mixture of all 15 ten modifications at the other positions (designated X). In a subsequent step (step 3), the different pools of ribozymes are tested for desired activity, phenotype, characteristic or attribute. For example, the testing may be determining *in vitro* rates of target nucleic acid cleavage for each pool, testing ribozyme-substrate binding affinities, testing nuclease resistance, determining pharmacodynamic properties, or determining which pool is most 20 efficacious in a cellular or animal model system. Following testing, a particular pool is identified as a desired variant (designated "Desired Variant-1") and the nucleotide or the analog present at the fixed position within the Desired Variant-1 is made constant (designated "Z") for all subsequent experiments; a single position within a ribozyme is therefore varied, *i.e.*, the variable nucleotide or analog at a single position, when all other 25 X positions are random, is identified within a ribozyme motif. Subsequently, new ribozyme pools (Classes 2, 3 *etc.*) are synthesized containing an equal mixture of all nucleotides or analogs at the remaining positions to be optimized except one fixed position and one or more constant positions. Again, a specific nucleotide or analog is "fixed" at a single position that is not randomized and the pools are assayed for a

particular phenotype or attribute (step 4). This process is repeated until all desired positions have been varied and screened. For example if three positions are chosen for optimization, the synthesis and testing will need to be repeated three times (3 Classes). In the first two Classes, pools will be synthesized; in the final Class, specific ribozymes will 5 be synthesized and tested. When the final position is analyzed (step 5), no random positions will remain and therefore only individual ribozymes are synthesized and tested. The resulting ribozyme or ribozymes (designated "second ribozyme motif") will have a defined chemical composition which will likely be distinct from the Starting Ribozyme motif (first ribozyme motif). This is a rapid method of screening for variability of one or 10 more positions within a ribozyme motif.

In another preferred embodiment, the invention involves screening of chemical modifications at one or more positions within a hammerhead ribozyme motif. More specifically, the invention involves variability in the catalytic core sequence of a hammerhead ribozyme. Particularly, the invention describes screening for variability of 15 positions 3, 4 and 7 within a hammerhead ribozyme. The invention also features screening for optimal loop II sequence in a hammerhead ribozyme.

In yet another preferred embodiment, the invention features a rapid method for screening accessible ribozyme cleavage sites within a target sequence. This method involves screening of all possible sequences in the binding arm of a ribozyme. The 20 sequence of the binding arms determines the site of action of certain ribozymes. The combinatorial approach can be used to identify desirable and/or accessible sites within a target sequence by essentially testing all possible arm sequences. The difficulty with this approach is that ribozymes require a certain number of base pairs (for example, for hammerhead ribozymes the binding arm length is approximately 12-16 nucleotides) in 25 order to bind functionally and sequence-specifically. This would require, for example 12-16 different groups of hammerhead ribozyme pools; 12-16 positions would have to be optimized which would require 12-16 different groups being synthesized and tested. Each pool would contain the four different nucleotides (A, C, U and G) or nucleotide

analogs ( $p = 4$  for nucleotides). It would be very time consuming to test each group, identify the best pool, synthesize another group of ribozyme pools with one additional position constant, and then repeat the procedure until all 12-16 groups had been tested. However it is possible to decrease the number of Classes by testing multiple positions 5 within a single Class. In this case, the number of pools within a Class equals the number of nucleotides or analogs in the random mixture (i.e.,  $n$ ) to the  $w$  power, where  $w$  equals the number of positions fixed in each Class. The number of Classes that need to be synthesized to optimize the final ribozyme equals the total number of positions to be optimized divided by the number of positions ( $w$ ) tested within each Class. The number 10 of pools in each Class =  $n^w$ . The number of Class = total number of positions / $w$ .

In another preferred embodiment, the invention features a rapid method of screening for new catalytic nucleic acid motifs by keeping the binding arms constant and varying one or more positions in a putative catalytic domain. Applicant describes a method to vary positions within the catalytic domain, without changing positions within 15 the binding arms, in order to identify new catalytic motifs. An example is illustrated in Figure 24. It is unclear how many positions are required to obtain a functional catalytic domain in a nucleic acid molecule, however it is reasonable to presume that if a large number of functionally diverse nucleotide analogs can be used to construct the pools, a relatively small number of positions could constitute a functional catalytic domain. This 20 may especially be true if analogs are chosen that one would expect to participate in catalysis (e.g., acid/base catalysts, metal binding, etc.). In the example illustrated, four positions (designated 1, 2, 3 and 4) are chosen. In the first step, ribozyme libraries (Class 1) are constructed: position 1 is fixed ( $F_1$ ) and positions 2, 3 and 4 are random ( $X_2$ ,  $X_3$  and  $X_4$ , respectively). In step 2, the pools (the number of pools tested depends on the number 25 of analogs used;  $n$ ) are assayed for activity. This testing may be performed *in vitro* or in a cellular or animal model. Whatever assay that is used, the pool with the desired characteristic is identified and libraries (class 2) are again synthesized with position 1 now constant ( $Z_1$ ) with the analog that was identified in class 1. In class 2, position 2 is fixed ( $F_2$ ) and positions 3 and 4 are random ( $X_3$  and  $X_4$ ). This process is repeated until

every position has been made constant and the chemical composition of the catalytic domain is determined. If the number of positions in the catalytic domain to be varied are large, then it is possible to decrease the number of Classes by testing multiple positions within a single Class. The number of pools within a Class equals the number of nucleotides or analogs in the random mixture (i.e., n) to the w power, where w equals the number of positions fixed in each Class. The number of Classes that need to be synthesized to optimize the final ribozyme equals the total number of positions to be optimized divided by the number of positions (w) tested within each Class. The number of pools in each Class=  $n^w$ . The number of Classes= total number of positions /w.

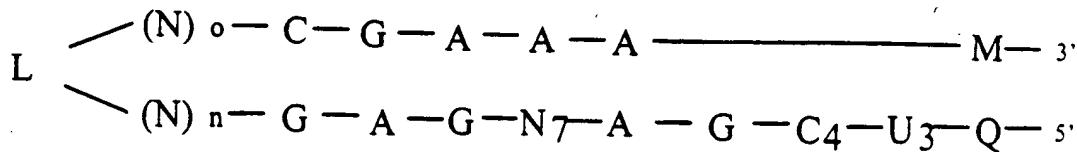
10 In a preferred embodiment a method for identifying variants of a nucleic acid catalyst is described comprising the steps of: a) selecting at least three (3) positions, preferably 3-12, specifically 4-10, within said nucleic acid catalyst to be varied with a predetermined group of different nucleotides, these nucleotides are modified or unmodified (non-limiting examples of nucleotides that can be used in this method are shown in Figure 15); b) synthesizing a first class of different pools of said nucleic acid catalyst, wherein the number of pools synthesized is equal to the number of nucleotides in the predetermined group of different nucleotides (for example if 10 different nucleotides are selected to be in the group of predetermined nucleotides then 10 different pools of nucleic acid catalysts have to be synthesized), wherein at least one of the positions to be varied in each pool comprises a defined nucleotide (fixed position; F) selected from the predetermined group of different nucleotides and the remaining positions to be varied comprise a random mixture of nucleotides (X positions) selected from the predetermined group of different nucleotides; c) testing the different pools of said nucleic acid catalyst under conditions suitable for said pools to show a desired attribute (including but not limited to improved cleavage rate, cellular and animal efficacy, nuclease stability, enhanced delivery, desirable localization) and identifying the pool with said desired attribute and wherein the position with the defined nucleotide (F) in the pool with the desired attribute is made constant (Z position) in subsequent steps; d) synthesizing a second class of different pools of nucleic acid catalyst, wherein at least one of the

positions to be varied in each of the second class of different pools comprises a defined nucleotide (F) selected from the predetermined group of different nucleotides and the remaining positions to be varied comprise a random mixture (X) of nucleotides selected from the predetermined group of different nucleotides (this second class of pools therefore has F, X and Z positions); e) testing the second class of different pools of said nucleic acid catalyst under conditions suitable for showing desired attribute and identifying the pool with said desired attribute and wherein the position with the defined nucleotide in the pool with the desired attribute is made constant (Z) in subsequent steps; and f) this process is repeated until every position selected in said nucleic acid catalyst to be varied is made constant.

In yet another preferred embodiment, a method for identifying novel nucleic acid molecules in a biological system is described, comprising the steps of: a) synthesizing a pool of nucleic acid catalyst with a substrate binding domain and a catalytic domain, wherein said substrate binding domain comprises a random sequence; b) testing the pools of nucleic acid catalyst under conditions suitable for showing a desired effect (such as inhibition of cell proliferation, inhibition of angiogenesis, modulation of growth and/or differentiation, and others) and identifying the catalyst with said desired attribute; c) using an oligonucleotide, comprising the sequence of the substrate binding domain of the nucleic acid catalyst showing said desired effect, as a probe, screening said biological system for nucleic acid molecules complementary to said probe; and d) isolating and sequencing said complementary nucleic acid molecules. These nucleic acid molecules identified using a nucleic acid screening method described above may be new gene sequences, or known gene sequences. The advantage of this method is that nucleic acid sequences, such as genes, involved in a biological process, such as differentiation, cell growth, disease processes including cancer, tumor angiogenesis, arthritis, cardiovascular disease, inflammation, restenosis, vascular disease and the like, can be readily identified.

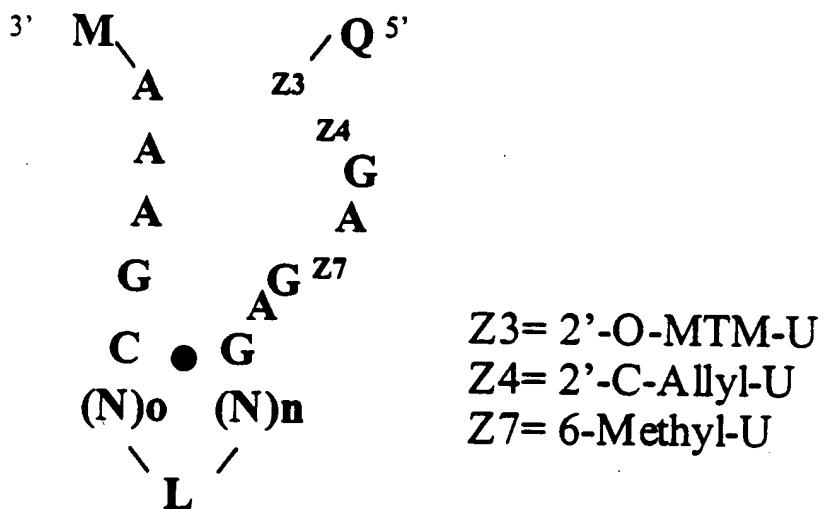
In a preferred embodiment, the invention features a nucleic acid molecule with catalytic activity having one of the formulae III-VII:

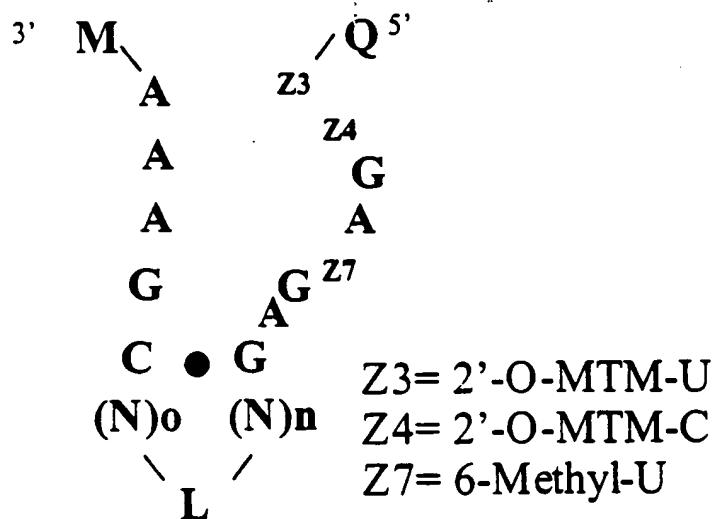
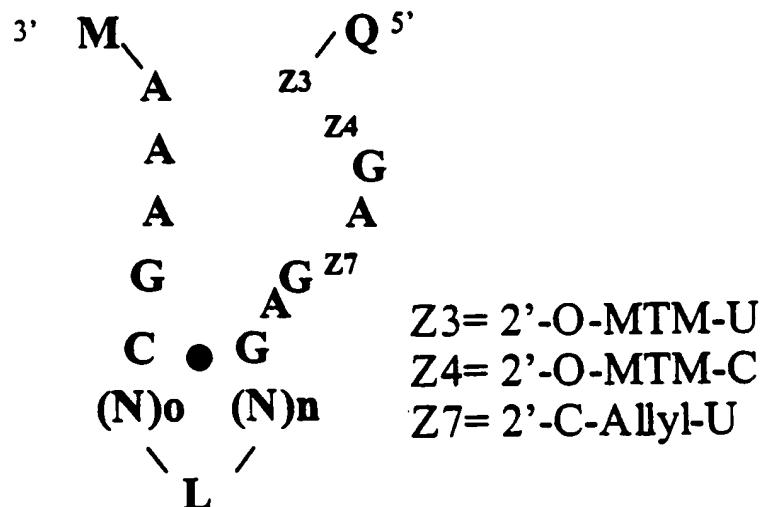
Formula III

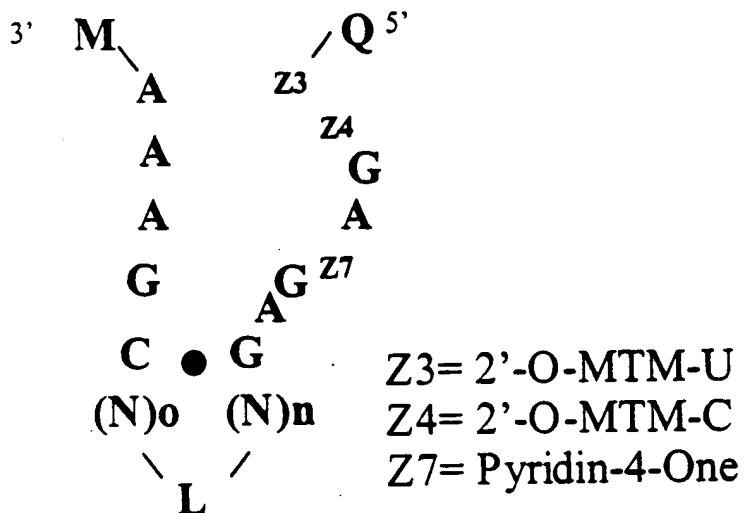


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Formula IV



Formula VFormula VI

Formula VII

In each of the above formulae, N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; M and Q are independently oligonucleotides of length sufficient to stably interact (e.g., by forming hydrogen bonds with complementary nucleotides in the target) with a target nucleic acid molecule (the target can be an RNA, DNA or RNA/DNA mixed polymers); preferably the length of Q is greater than or equal to 3 nucleotides and the length of M is preferably greater than or equal to 5 nucleotides; o and n are integers greater than or equal to 1 and preferably less than about 100, wherein if (N)o and (N)n are nucleotides, (N)o and (N)n are optionally able to interact by hydrogen bond interaction; L is a linker which may be present or absent (i.e., the molecule is assembled from two separate molecules), but when present, is a nucleotide and/or a non-nucleotide linker, which may be a single-stranded and/or double-stranded region; and   represents a chemical linkage (e.g., a phosphate ester linkage, amide linkage or others known in the art). 2'-O-MTM-U and 2'-O-MTM-C refers to 2'-O-methylthiomethyl uridine and 2'-O-methylthiomethyl-cytidine, respectively. A, C, U and G represent adenosine, cytidine, uridine and guanosine nucleotides, respectively. The nucleotides in the formulae are unmodified or modified at the sugar, base, and/or phosphate portions as known in the art.

In yet another embodiment, the nucleotide linker (L) is a nucleic acid aptamer, such as an ATP aptamer, HIV Rev aptamer (RRE), HIV Tat aptamer (TAR) and others (for a review see Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; and Szostak & Ellington, 1993, in *The RNA World*, ed. Gesteland and Atkins, pp 511, CSH Laboratory Press). A "nucleic acid aptamer" as used herein is meant to indicate nucleic acid sequence capable of interacting with a ligand. The ligand can be any natural or a synthetic molecule, including but not limited to a resin, metabolites, nucleosides, nucleotides, drugs, toxins, transition state analogs, peptides, lipids, proteins, aminoacids, nucleic acid molecules, hormones, carbohydrates, receptors, cells, viruses, bacteria and others.

In yet another embodiment, the non-nucleotide linker (L) is as defined herein.

In particular, the invention features modified ribozymes having a base substitution selected from pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyluracil, dihydrouracil, naphthyl, 6-methyl-uracil and aminophenyl.

In yet another embodiment, the non-nucleotide linker (L) is as defined herein. The term "non-nucleotide" as used herein include either abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, or polyhydrocarbon compounds. Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, 18:6353 and *Nucleic Acids Res.* 1987, 15:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, 113:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, 113:5109; Ma *et al.*, *Nucleic Acids Res.* 1993, 21:2585 and *Biochemistry* 1993, 32:1751; Durand *et al.*, *Nucleic Acids Res.* 1990, 18:6353; McCurdy *et al.*, *Nucleosides & Nucleotides* 1991, 10:287; Jschke *et al.*, *Tetrahedron Lett.* 1993, 34:301; Ono *et al.*, *Biochemistry* 1991, 30:9914; Arnold *et al.*, International Publication No. WO 89/02439; Usman *et al.*, International Publication No. WO 95/06731; Dudycz *et al.*, International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, 113:4000, all hereby incorporated by reference herein. Thus, in a preferred embodiment, the invention features a nucleic acid catalyst having one or more non-nucleotide moieties, and having enzymatic

activity to cleave an RNA or DNA molecule. By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is 5 abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine. The terms "abasic" or "abasic nucleotide" as used herein encompass sugar moieties lacking a base or having other chemical groups in place of base at the 1' position.

10 In preferred embodiments, the enzymatic nucleic acid includes one or more stretches of RNA, which provide the enzymatic activity of the molecule, linked to the non-nucleotide moiety. The necessary RNA components are known in the art, see, e.g., Usman, *supra*. By RNA is meant a molecule comprising at least one ribonucleotide residue.

15 As the term is used in this application, non-nucleotide-containing enzymatic nucleic acid means a nucleic acid molecule that contains at least one non-nucleotide component which replaces a portion of a ribozyme, *e.g.*, but not limited to, a double-stranded stem, a single-stranded "catalytic core" sequence, a single-stranded loop or a single-stranded recognition sequence. These molecules are able to cleave (preferably, repeatedly cleave) separate RNA or DNA molecules in a nucleotide base sequence 20 specific manner. Such molecules can also act to cleave intramolecularly if that is desired. Such enzymatic molecules can be targeted to virtually any RNA transcript.

25 The specific nucleic acid catalysts described in the instant application are not limiting in the invention and those skilled in the art will recognize that all that is important in a nucleic acid catalyst of this invention is that it has a specific substrate binding site (*e.g.*, M and/or Q of Formulae III-VII above) which is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart a nucleic acid cleaving activity to the molecule.

Vector Expression of Enzymatic Nucleic Acid

The nucleic acid catalysts of the instant invention can be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985 *Science* 229, 345; McGarry and Lindquist, 1986 *Proc. Natl. Acad. Sci. USA* 83, 399; Scanlon *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet *et al.*, 1992 *Antisense Res. Dev.*, 2, 3-15; Dropulic *et al.*, 1992 *J. Virol.*, 66, 1432-41; Weerasinghe *et al.*, 1991 *J. Virol.*, 65, 5531-4; Ojwang *et al.*, 1992 *Proc. Natl. Acad. Sci. USA* 89, 10802-6; Chen *et al.*, 1992 *Nucleic Acids Res.*, 20, 4581-9; Sarver *et al.*, 1990 *Science* 247, 1222-1225; Thompson *et al.*, 10 1995 *Nucleic Acids Res.* 23, 2259; Good *et al.*, 1997, *Gene Therapy*, 4, 45; all of the references are hereby incorporated in their totality by reference herein). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a ribozyme (Draper *et al.*, PCT WO 93/23569, 15 and Sullivan *et al.*, PCT WO 94/02595; Ohkawa *et al.*, 1992 *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira *et al.*, 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura *et al.*, 1993 *Nucleic Acids Res.*, 21, 3249-55; Chowtira *et al.*, 1994 *J. Biol. Chem.* 269, 25856; all of the references are hereby incorporated in their totality by reference herein).

In another aspect of the invention, nucleic acid catalysts that cleave target molecules 20 are expressed from transcription units (see for example Figure 11) inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the ribozymes are delivered as described above, and persist 25 in target cells. Alternatively, viral vectors may be used that provide for transient expression of ribozymes. Such vectors might be repeatedly administered as necessary. Once expressed, the ribozymes cleave the target mRNA. The active ribozyme contains an enzymatic center or core equivalent to those in the examples, and binding arms able to bind target nucleic acid molecules such that cleavage at the target site occurs. Other

sequences may be present which do not interfere with such cleavage. Delivery of ribozyme expressing vectors could be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction 5 into the desired target cell (for a review see Couture and Stinchcomb, 1996, *TIG.*, 12, 510).

In a preferred embodiment, an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid catalyst of the instant invention is disclosed. The nucleic acid sequence encoding the nucleic acid catalyst of the instant invention is 10 operable linked in a manner which allows expression of that nucleic acid molecule.

In one embodiment, the expression vector comprises: a transcription initiation region (e.g., eukaryotic pol I, II or III initiation region); b) a transcription termination region (e.g., eukaryotic pol I, II or III termination region); c) a gene encoding at least one of the nucleic acid catalyst of the instant invention; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. The vector may optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the gene encoding the nucleic acid catalyst of the invention; and/or an intron (intervening sequences).

20 Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA 25 polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990 *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993 *Nucleic Acids Res.*, 21, 2867-72; Lieber et al., 1993 *Methods Enzymol.*, 217, 47-66; Zhou et al., 1990 *Mol. Cell. Biol.*, 10,

4529-37). Several investigators have demonstrated that ribozymes expressed from such promoters can function in mammalian cells (e.g., Kashani-Sabet et al., 1992 *Antisense Res. Dev.*, 2, 3-15; Ojwang et al., 1992 *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen et al., 1992 *Nucleic Acids Res.*, 20, 4581-9; Yu et al., 1993 *Proc. Natl. Acad. Sci. U S A*, 90, 5 6340-4; L'Huillier et al., 1992 *EMBO J.* 11, 4411-8; Lisziewicz et al., 1993 *Proc. Natl. Acad. Sci. U. S. A.*, 90, 8000-4; Thompson et al., 1995 *Nucleic Acids Res.* 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired 10 RNA molecules such as ribozymes in cells (Thompson et al., *supra*; Couture and Stinchcomb, 1996, *supra*; Noonberg et al., 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg et al., US Patent No. 5,624,803; Good et al., 1997, *Gene Ther.* 4, 45; Beigelman et al., International PCT Publication No. WO 96/18736; all of these publications are incorporated by reference herein. Examples of transcription units suitable for expression 15 of ribozymes of the instant invention are shown in Figure 11. The above ribozyme transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, 1996, 20 *supra*).

In a preferred embodiment an expression vector comprising nucleic acid sequence encoding at least one of the catalytic nucleic acid molecule of the invention, in a manner which allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination 25 region; c) a gene encoding at least one said nucleic acid molecule; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another preferred embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; d) a gene encoding at least one

said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In yet another embodiment the expression 5 vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) a gene encoding at least one said nucleic acid molecule; and wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another embodiment, the expression vector comprises: a) a transcription initiation region; 10 b) a transcription termination region; c) an intron; d) an open reading frame; e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

15 Delivery of Nucleic Acid Catalysts:

In a preferred embodiment, the nucleic acid catalysts are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to smooth muscle cells. The RNA or RNA complexes can be locally administered to relevant tissues through the use of a catheter, infusion pump or stent, with or without their 20 incorporation in biopolymers. Using the methods described herein, other nucleic acid catalysts that cleave target nucleic acid may be derived and used as described above. Specific examples of nucleic acid catalysts of the instant invention are provided below in the Tables and figures.

Sullivan, *et al.*, *supra*, describes the general methods for delivery of enzymatic 25 RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications,

ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination is locally delivered by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or 5 joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme delivery and administration are provided in Sullivan *et al.*, *supra* and Draper *et al.*, *supra* which have been incorporated by reference herein.

10 The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, ammonium, sodium, calcium, magnesium, lithium, and potassium salts.

15 A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or patient, preferably a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation to reach a target cell (*i.e.*, a cell to which the negatively charged polymer is desired to be delivered to). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms which prevent the 20 composition or formulation from exerting its effect.

25 By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitations: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged polymers, *e.g.*, NTP's, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant

invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation which can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of the drug to 5 target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as the cancer cells.

The invention also features the use of the a composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer an method for 10 increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al.* *Chem. Rev.* 1995, **95**, 2601-2627; Ishiwata *et al.*, *Chem. Pharm. Bull.* 1995, **43**, 1005-1011). Such liposomes have been shown to accumulate 15 selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al.*, *Science* 1995, **267**, 1275-1276; Oku *et al.*, 1995, *Biochim. Biophys. Acta*, **1238**, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of drugs, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al.*, *J. Biol. Chem.* 1995, **42**, 24864-24870; Choi *et al.*, International PCT Publication No. WO 20 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390; Holland *et al.*, International PCT Publication No. WO 96/10392; all of these are incorporated by reference herein). Long-circulating liposomes are also likely to protect drugs from nucleic acid degradation to a greater extent compared to cationic liposomes, based on their 25 ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen. All of these references are incorporated by reference herein.

The present invention also includes compositions prepared for storage or administration which include a pharmaceutically effective amount of the desired

compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985) hereby incorporated by reference herein. For example, preservatives, 5 stabilizers, dyes and flavoring agents may be provided. *Id.* at 1449. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) 10 of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors which those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active 15 ingredients is administered dependent upon potency of the negatively charged polymer. In a one aspect, the invention provides nucleic acid catalysts that can be delivered exogenously to specific cells as required.

Local ribozyme administration offers the advantages of achieving high tissue concentrations of ribozymes and limiting their exposure to catabolic and excretory 20 mechanisms. Although local routes of administration provide access to pathologies involving a number of organ systems, systemic administration would make ribozyme treatment of several other major human diseases feasible.

It has been demonstrated that certain tissues accumulate oligonucleotides and/or 25 oligonucleotide formulations following systemic administration. These tissues include sites of inflammation (Wu *et al.* 1993, *Cancer Res.* 53: 3765-3767), solid tumors (Yuan *et al.* 1994, *Cancer Res.* 54: 3352-3356), kidney (Cossum *et al.* 1993, *J. Pharmacol. and Exp. Ther.* 267: 1181-1190), brain (Wu *et al.* 1996, *J. Pharmacol. Exp. Ther.* 276: 206-11) and those rich in reticulo-endothelial cells (liver, spleen, lymphatics; Litzinger *et al.*

1994, *Biochim. Biophys. Acta* 1190: 99-107; Agrawal *et al.* 1991, *Proc. Natl. Acad. Sci. USA* 88: 7595-7599; Agrawal *et al.* 1995, *Clin. Pharmacology* 28: 7-16; Sands *et al.* 1994, *Molecular Pharmacol.* 45: 932-943; Saijo *et al.* 1994, *Oncology Research* 6: 243-249).

5 The kidney, as well as organs of the reticulo-endothelial system (RES), are mainly responsible for clearance of ribozymes following intravenous (*i.v.*) administration. Diseases involving these tissues are good candidates for systemic ribozyme therapy by virtue of their tendency to accumulate ribozymes.

In one preferred embodiment, the invention features method of treating inflammation 10 using ribozymes. Inflammatory processes underlie the pathology of a large number of human diseases. Many of these processes can be blocked by inhibiting the expression of inflammatory mediators and/or their receptors (Cohen *et al.* 1995, *Am. J. Med.* 99: 45S-52S). Systemic administration of monoclonal antibodies specific to these mediators have been shown to be efficacious in animal models of rheumatoid arthritis, inflammatory 15 bowel disease, and acute respiratory distress syndrome (Arend *et al.* 1990, *Arthritis and Rheumatism* 33: 305-315). One potential way for systemic administration of ribozymes to impact systemic inflammatory disease is through inhibition of TNF- $\alpha$  production by macrophages. TNF- $\alpha$  has been shown to participate in a variety of inflammatory processes and is produced mainly by macrophages which are known to accumulate cationic lipid- 20 formulated ribozymes (Masahiro *et al.* 1990, *J. Immunology* 144: 1425-1431). Anti-mouse TNF- $\alpha$  ribozymes were effective in cell culture, thus, it may be possible that systemic delivery of ribozymes by a liposome formulation could be an effective therapeutic in the above mentioned inflammatory disease states.

In another preferred embodiment, the invention features methods of treating diseases 25 involving RES using ribozymes. A number of studies have shown that systemically administered oligonucleotides distribute to RES tissues (liver, spleen and lymphatics). Several studies with cationic lipid complexed oligonucleotides have also shown specific biodistribution to these. Pathology involving the RES includes a number of infectious

diseases of major importance, such as human immunodeficiency virus (HIV), mycobacterium infections including tuberculosis (TB), avium, and leprae (leprosy). These diseases are all associated with, for example, overproduction of interleukin-10 (IL-10), a potent immunosuppressive cytokine (Barnes *et al.* 1993, *Infect. Immun.* 61: 3482-9). Some of these infections can potentially be ameliorated by administration of 5 neutralizing antibodies to IL-10.

In yet another preferred embodiment, the invention features method of treating cancer using ribozymes. As evidence of the potential use of systemic oligonucleotides as anticancer agents, antisense phosphorothioates have been have been reported to exhibit 10 antitumor efficacy in a murine model of Burkitt's lymphoma (Huang *et al.* 1995, *Mol. Med.* 1: 647-658). The molecular targets of systemic antineoplastic ribozymes could include oncogenes, protooncogenes, or angiogenic factors and receptors. Although the link between oncogenes and tumorigenesis is now well established, the specific mutations 15 that lead to activation of a proto-oncogene can be widely diverse. Upregulation of protooncogene products is also common in human cancer. Reducing the levels of these gene products may be beneficial in treatment of cancer. In addition, since many tumors are highly vascularized, angiogenic factors or receptors may provide good alternate or adjunct targets to oncogenes for the therapy of solid tumors and their metastases. Applicant, in a non-limiting example *infra*, show ribozymes targeting angiogenic 20 mediators.

The potential number of molecular targets in cancer is quite large. Among these targets are oncogenes, protooncogenes, metalloproteinases, growth factors, and angiogenic factors. However, a common denominator in many forms of metastatic solid tumors is extensive vascularization of the tumor. As tumors exceed about 1 mm in 25 diameter, they require neovascularization for continued growth (Gimbrone *et al.*, 1972, *J. Exp. Med.*, 136, 261). In addition, the appearance of new blood vessels within a tumor correlates with the initiation of the process of metastasis (Martiny-Baron and Marmé, 1995). It is possible that by using a systemically administered ribozyme targeting a key

player in the process of angiogenesis would reduce both primary tumor growth, tumor progression and tumor metastasis.

"Angiogenesis" refers to formation of new blood vessels from existing blood vessels which is an essential process in reproduction, development and wound repair.

5 "Tumor angiogenesis" refers to the induction of the growth of blood vessels from surrounding tissue into a solid tumor. Tumor growth and tumor metastasis are dependent on angiogenesis (for a review see Folkman, 1985, *Nature Med.* 1: 27-31; Folkman 1990 *J. Natl. Cancer Inst.*, 82, 4; Folkman and Shing, 1992 *J. Biol. Chem.* 267, 10931).

10 "Tumor metastasis" refers to the transfer and/or migration of tumor cells, originating from a primary tumor, from one part of the body or organ to another. Most malignant tumors have the capacity to metastasize.

"Tumor" refers to a new growth of tissue wherein the cells multiply, divide and grow uncontrolled.

15 In a preferred embodiment, the invention features a method of treating non-hepatic ascites using ribozymes. Nonhepatic ascites or peritoneal fluid accumulation resulting from abdominal cancer and ovarian hyperstimulation syndrome (OHSS) can result in significant fluid loss from the intravascular space and hypovolemia. If ascites volumes are large, abdominal pain, hypovolemic hypotension, electrolyte abnormalities and respiratory difficulties can ensue. Thus, if ascites is left untreated, it can be life threatening. Evidence is now accumulating that nonhepatic ascites may be induced, at least in part, by vascular endothelial growth factor (VEGF). For this reason, nonhepatic ascites may be a potential therapeutic indication for ribozymes directed against vascular endothelial growth factor (VEGF) receptors delivered either systemically or regionally to the peritoneum.

25 Ovaries can be overstimulated by hormonal therapy during fertility treatment. As a result, women can experience ovarian hyperstimulation syndrome which is associated

with grossly enlarged ovaries and extreme ascites fluid accumulation. This fluid accumulation is thought to be induced by the release of a vascular permeability agent which may interact with vessels of the peritoneal cavity leading to plasma extravasation. Abramov and co-workers (1997, *Fertil. Steril.* 67: 261) have shown that plasma VEGF 5 levels are elevated in OHSS and return to normal upon resolution of the syndrome. An earlier study has shown that VEGF is elevated in the serum and follicular fluid of OHSS patients and that the source of this VEGF may be the luteinizing granulosa cells of the ovary (Krasnow et al., 1996, *Fertil. Steril.* 65: 552). McClure et al. (1994, *Lancet* 344, 235) concluded that VEGF is the key mediator of OHSS ascites production since rhVEGF 10 increases OHSS ascites but not liver ascites and that this increase is reversible by rhVEGF antiserum. Thus, reducing the expression of VEGF receptors in the vasculature of the peritoneum may have a therapeutic benefit in OHSS by substantially reducing OHSS- stimulated ascites production. Since VEGF can interact with VEGF receptors on vessels throughout the peritoneum from ovarian release of VEGF into systemic circulation, 15 systemic treatment may represent the best option for treating this syndrome.

**Malignant ascites:** Another form of ascites can be induced by malignancies of the peritoneum including breast, pancreatic, uterine and colorectal cancers. It is thought that certain cancers produce factors which influence peritoneal vascular permeability leading to plasma extravasation (Garrison et al., 1986; *Ann. Surg.* 203: 644; Garrison et al., 1987, 20 *J. Surg. Res.* 42: 126; Nagy et al., 1993, *Cancer Res.* 53: 2631). Several solid tumors including some colorectal and breast carcinomas are known to secrete VEGF to recruit blood vessels for sustained growth and metastasis. This secreted VEGF may also serve to increase local vasculature permeability. In support of this hypothesis, Nagy et al. (*supra*) 25 showed in mice that peritoneal fluid resulting from MOT and TTA3/St carcinomas contained elevated levels of VEGF whose concentration correlated directly with fluid accumulation and development of hyperpermeable microvessels. Therefore, ribozymes directed against VEGF receptors administered systemically may impact both the tumor growth and metastases of VEGF secreting tumors as well as ascites induced by VEGF interacting with the vasculature of the peritoneum.

Strategies for Systemic Delivery*Methods to enhance tissue accumulation*

Tissue accumulation of ribozymes can be improved by formulation, conjugation, or further chemical stabilization of the ribozyme. Elimination due to glomerular filtration 5 can be slowed by increasing the apparent molecular weight of the ribozyme, e.g., by liposome encapsulation or bioconjugation to PEG. Applicant has observed that the rate of catabolism can be slowed by a factor of 100 and lung accumulation increased 500 fold by formulation with DMRIE/DOPE reagents. Liposomal encapsulation is likely to have a similar effect on the rate of catabolism. The rate of clearance into non-target tissues could 10 also be reduced by encapsulation into liposomes, provided that the liposomes were surface modified with PEG such that RES clearance were avoided. Increasing the rate of uptake by target tissues can also be enhanced, for example, by conjugation of cholesterol to the ribozymes. Applicant has also observed that in tissues of the RES, accumulation has been increased several hundred fold by complexation with a cationic lipid carrier.

15 *Sustained release as a means to increase exposure*

Sustained or continuous delivery devices, such as ALZET® osmotic mini-pumps, may also enhance accumulation in target tissues by increasing exposure relative to bolus i.v. administration. Sustained delivery from ALZET® pumps has been shown to be an effective way of administering a phosphorothioate antisense molecule for inhibition of 20 tumor growth in mice (Huang *et al.* 1995, *supra*). Applicant has observed that the rate of ribozyme catabolism in and rate of clearance from the circulation is concentration dependent and may relate to the equilibrium plasma protein binding of the ribozyme. Phosphorothioate DNA is rapidly cleared from circulation when its concentration exceeds the plasma protein binding constant, as is the case after i.v. bolus administration. 25 Osmotic pumps administer oligonucleotides at a slower and constant rate, and therefore may maintain plasma levels near the equilibrium binding capacity. This would result in less of the administered dose being lost to glomerular filtration (elimination) and hepatic

extraction (catabolism); more of the administered dose may be available for uptake into target tissues.

### Animal Models

#### *Use of murine models*

5 For a typical systemic study involving 10 mice (20 g each) per dose group, 5 doses (1, 3, 10, 30 and 100 mg/kg daily over 14 days continuous administration), approximately 400 mg of ribozyme, formulated in saline would be used. A similar study in young adult rats (200 g) would require over 4 g. Parallel pharmacokinetic studies may involve the use of similar quantities of ribozymes further justifying the use of murine models.

10 ***Ribozymes and Lewis lung carcinoma and B-16 melanoma murine models***

Identifying a common animal model for systemic efficacy testing of ribozymes is an efficient way of screening ribozymes for systemic efficacy.

The Lewis lung carcinoma and B-16 murine melanoma models are well accepted models of primary and metastatic cancer and are used for initial screening of anti-cancer. 15 These murine models are not dependent upon the use of immunodeficient mice, are relatively inexpensive, and minimize housing concerns. Both the Lewis lung and B-16 melanoma models involve subcutaneous implantation of approximately  $10^6$  tumor cells from metastatically aggressive tumor cell lines (Lewis lung lines 3LL or D122, LLc-LN7; B-16-BL6 melanoma) in C57BL/6J mice. Alternatively, the Lewis lung model can be 20 produced by the surgical implantation of tumor spheres (approximately 0.8 mm in diameter). Metastasis also may be modeled by injecting the tumor cells directly *i.v.*. In the Lewis lung model, microscopic metastases can be observed approximately 14 days following implantation with quantifiable macroscopic metastatic tumors developing within 21-25 days. The B-16 melanoma exhibits a similar time course with tumor 25 neovascularization beginning 4 days following implantation. Since both primary and metastatic tumors exist in these models after 21-25 days in the same animal, multiple

measurements can be taken as indices of efficacy. Primary tumor volume and growth latency as well as the number of micro- and macroscopic metastatic lung foci or number of animals exhibiting metastases can be quantitated. The percent increase in lifespan can also be measured. Thus, these models would provide suitable primary efficacy assays for 5 screening systemically administered ribozymes/ribozyme formulations.

In the Lewis lung and B-16 melanoma models, systemic pharmacotherapy with a wide variety of agents usually begins 1-7 days following tumor implantation/inoculation with either continuous or multiple administration regimens. Concurrent pharmacokinetic studies can be performed to determine whether sufficient tissue levels of ribozymes can 10 be achieved for pharmacodynamic effect to be expected. Furthermore, primary tumors and secondary lung metastases can be removed and subjected to a variety of *in vitro* studies (i.e., target mRNA reduction).

#### *Anti-VEGF receptor ribozymes*

Sustained tumor growth and metastasis depend upon angiogenesis. In fact, the 15 appearance of vessels in a growing tumor is correlated with the beginning of metastatic potential. Several studies have shown that antiangiogenic agents alone or in combination with cytotoxic agents reduce lung metastases and/or primary tumor volume in the Lewis lung and B-16 melanoma models (Borgstrom *et al.* 1995, *Anticancer Res.* 15: 719-728; Kato *et al.* 1994, *Cancer Res.* 54: 5143-5147; O'Reilly *et al.* 1994, *Cell* 79: 315-328; 20 Sato *et al.* 1995, *Jpn. J. Cancer Res.* 86: 374-382).

A major factor implicated in the induction of solid tumor angiogenesis is vascular 25 endothelial growth factor (VEGF; Folkman, 1995, *supra*). Several human tumors have been shown to synthesize and secrete. With regard to treating lung metastasis, VEGF and VEGF receptors of both subtypes and their expression are upregulated in the lung under conditions of hypoxia (Tuder *et al.* 1994, *J. Clin. Invest.* 95: 1798-1807). This may lead to neovascularization which provides the means by which tumor cells gain access to

circulation (Mariny-Baron and Marmé, 1995). Thus, VEGF and its receptors may be important targets in the treatment of metastatic disease.

Applicant has shown that a catalytically active ribozyme targeting *flt-1* RNA inhibits VEGF-induced neovascularization in a dose-dependent manner in a rat corneal model of angiogenesis. Testing with cytotoxic agents in combination with antiangiogenic ribozymes may also prove useful.

#### *Anti-K- and H-ras ribozymes*

Mutations involving *ras* underlie a number of human cancers. *Ras* also plays a role in metastatic potential (Shekhar and Miller, 1994, *Invasion Metastasis* 14: 27-37) and may do so, in part, by influencing endothelial cell migration (Fox *et al.* 1994, *Oncogene* 9: 3519-26). With regard to lung cancer, *ras* has been shown to induce abnormal mitoses in lung fibroblasts (Lyubuski *et al.* 1994, *Cytobios* 80: 161-178) and is a clinical marker in non-small cell lung tumors (Niklinski and Furman, 1995, *Eur. J. Cancer Prev.* 4: 129-138). Studies in cells cultured from human small cell lung tumor xenografts demonstrated overexpression of K-*ras* (Arvelo *et al.* 1994, *Anticancer Res.* 14: 1893-1901). This evidence provides ample support for the systemic testing of ribozymes directed against H- and K-*ras* in the murine cancer models (primary and secondary metastasis) discussed above.

Four of the current synthetic ribozymes directed against human K-*ras* will cleave homologous mouse K-*ras* targets at four sites and inhibit cultured rat aortic smooth muscle cell proliferation.

#### *Anti-c-fos ribozymes*

The protein product of the proto-oncogene *c-fos* is a nuclear transcription factor which is involved in tumorigenesis. In support of the possible use of systemically administered ribozymes directed against *c-fos*, null mouse mutations of *c-fos* have been shown to result in viable mice. Using this mouse model, it has been shown that *c-fos* is

important in malignant conversion of papillomas. Additionally, *c-fos* has been shown to up-regulate tumor metalloproteinases (Schonthal *et al.* 1988, *Cell* 54: 325-334). It is possible that *c-fos* may play a role in tumor angiogenesis as evidenced by VEGF mRNA levels being significantly reduced in *c-fos* deficient tumors. It has also been shown that *c-fos* is highly expressed in some B-16 cell and human melanoma cell lines (Kroumpouzos *et al.* 1994, *Pigment Cell Res.* 7: 348-353; Nakayama *et al.* 1995, *J. Dermatol.* 22: 549-559; Peris *et al.* 1991, *Arch. Dermatol. Res.* 283: 500-505). The expression of *c-fos* may be directly proportional to metastatic potential in B-16 melanoma cell lines. With this evidence, it is reasonable to conclude that *c-fos* represents a suitable systemic ribozyme target in either the Lewis lung, B-16 melanoma, or human melanoma models.

Delivery of ribozymes and ribozyme formulations in the Lewis lung model

Several ribozyme formulations, including cationic lipid complexes which may be useful for inflammatory diseases (e.g., DIMRIE/DOPE, *etc.*) and RES evading liposomes which may be used to enhance vascular exposure of the ribozymes, are of interest in cancer models due to their presumed biodistribution to the lung. Thus, liposome formulations can be used for delivering ribozymes to sites of pathology linked to an angiogenic response.

The sequences of the ribozymes that are chemically synthesized, useful in this study, are non-limiting examples. Those in the art will recognize that these sequences are representative only of many more such sequences where the enzymatic portion of the ribozyme (all but the binding arms) is altered to affect activity. For example, stem-loop II sequence of hammerhead ribozymes can be altered (substitution, deletion, and/or insertion) to contain any sequences provided a minimum of two base-paired stem structure can form. Similarly, stem-loop IV sequence of hairpin ribozymes listed in Tables IV (5'-CACGUUGUG-3') can be altered (substitution, deletion, and/or insertion) to contain any sequence, provided a minimum of two base-paired stem structure can form. Preferably, no more than 200 bases are inserted at these locations. The sequences listed in Tables III and IV may be formed of ribonucleotides or other nucleotides or non-

nucleotides. Such ribozymes (which have enzymatic activity) are equivalent to the ribozymes described specifically in the Tables.

Target sites

Targets for useful ribozymes can be determined as disclosed in Draper *et al.*, WO 93/23569; Sullivan *et al.*, WO 93/23057; Thompson *et al.*, WO 94/02595; Draper *et al.*, WO 95/04818; McSwiggen *et al.*, US Patent No. 5,525,468 and hereby incorporated by reference herein in totality. Rather than repeat the guidance provided in those documents here, below are provided specific examples of such methods, not limiting to those in the art. Ribozymes to such targets are designed as described in those applications and 10 synthesized to be tested *in vitro* and *in vivo*, as also described. Such ribozymes can also be optimized and delivered as described therein.

The sequence of human *c-raf* mRNAs were screened for optimal ribozyme target sites using a computer folding algorithm. Hammerhead or hairpin ribozyme cleavage sites were identified. These sites are shown in Tables XII-XIX (All sequences are 5' to 3' in the tables) The nucleotide base position is noted in the Tables as that site to be cleaved by the designated type of ribozyme. The nucleotide base position is noted in the Tables 15 as that site to be cleaved by the designated type of ribozyme.

Because Raf RNAs are highly homologous in certain regions, some ribozyme target sites are also homologous (see Table XVIII and XIX). In this case, a single ribozyme 20 will target different classes of Raf RNA. The advantage of one ribozyme that targets several classes of Raf RNA is clear, especially in cases where one or more of these RNAs may contribute to the disease state.

Hammerhead or hairpin ribozymes were designed that could bind and were 25 individually analyzed by computer folding (Jaeger *et al.*, 1989 *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the ribozyme sequences fold into the appropriate secondary structure. Those ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding

arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA. Ribozymes of the hammerhead or hairpin motif were designed to anneal to various sites in the mRNA message. The binding arms are complementary to the target site sequences described 5 above.

#### Examples

The following are non-limiting examples showing the selection, isolation, synthesis and activity of enzymatic nucleic acids of the instant invention.

The following examples demonstrate the selection of ribozymes that cleave c-raf RNA. The methods described herein represent a scheme by which ribozymes may be derived that cleave other RNA targets required for cell division. Also provided is a description of how such ribozymes may be delivered to cells. The examples demonstrate that upon delivery, the ribozymes inhibit cell proliferation in culture and modulate gene expression *in vivo*. Moreover, significantly reduced inhibition is observed if mutated 10 ribozymes that are catalytically inactive are applied to the cells. Thus, inhibition requires 15 ribozymes that are catalytically active.

#### Example 1: Identification of Potential Ribozyme Cleavage Sites in Human c-raf RNA

The sequence of human c-raf RNA was screened for accessible sites using a computer folding algorithm. Regions of the mRNA that did not form secondary folding 20 structures and contained potential hammerhead and/or hairpin ribozyme cleavage sites were identified. The sequences of these cleavage sites are shown in tables XII-XIX.

#### Example 2: Selection of Ribozyme Cleavage Sites in Human c-raf RNA

To test whether the sites predicted by the computer-based RNA folding algorithm corresponded to accessible sites in c-raf RNA, 20 hammerhead sites were selected for 25 analysis. Ribozyme target sites were chosen by analyzing genomic sequences of human c-raf (GenBank Accession No. X03484; Bonner *et al.*, 1986, *Nucleic Acids Research*, 14,

1009-1015) and prioritizing the sites on the basis of folding. Hammerhead ribozymes were designed that could bind each target (see Figure 1) and were individually analyzed by computer folding (Christoffersen *et al.*, 1994 *J. Mol. Struc. Theochem.*, 311, 273; Jaeger *et al.*, 1989, *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the ribozyme 5 sequences fold into the appropriate secondary structure. Those ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core were eliminated from consideration. As noted below, varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA. Ribozyme target sites within A-Raf were chosen 10 by analyzing genomic sequences of human A-raf-1 (GenBank Accession No. X04790; Beck *et al.*, 1987, *Nucleic Acids Research*, 115, 595-609). Ribozyme target sites within B-Raf were chosen by analyzing genomic sequences of human B-raf-1 (GenBank Accession No. M95712 M95720 X54072; Sitanandam *et al.*, 1990, *Oncogene*, 5, 1775-1780).

15 **Example 3: Chemical Synthesis and Purification of Ribozymes for Efficient Cleavage of c-raf RNA**

Ribozymes of the hammerhead or hairpin motif were designed to anneal to various sites in the RNA message. The binding arms are complementary to the target site sequences described above. The ribozymes were chemically synthesized. The method of 20 synthesis used followed the procedure for normal RNA synthesis as described in Usman *et al.*, (1987 *J. Am. Chem. Soc.*, 109, 7845), Scaringe *et al.*, (1990 *Nucleic Acids Res.*, 18, 5433) and Wincott *et al.*, *supra*, and made use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. The average stepwise coupling yields were >98%.

25 Inactive ribozymes were synthesized by substituting a U for G5 and a U for A14 (numbering from Hertel *et al.*, 1992 *Nucleic Acids Res.*, 20, 3252). Hairpin ribozymes were synthesized in two parts and annealed to reconstruct the active ribozyme (Chowrim and Burke, 1992 *Nucleic Acids Res.*, 20, 2835-2840). Ribozymes were also synthesized

from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51). Ribozymes were modified to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-flouro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34).

5      Ribozymes were purified by gel electrophoresis using general methods or were purified by high pressure liquid chromatography (HPLC; See Wincott et al., *supra*; the totality of which is hereby incorporated herein by reference) and were resuspended in water. The sequences of the chemically synthesized ribozymes used in this study are shown below in **Table XII-XIX**.

10     Example 4: Ribozyme Cleavage of c-raf RNA Target *in vitro*

Ribozymes targeted to the human c-raf RNA are designed and synthesized as described above. These ribozymes can be tested for cleavage activity *in vitro*, for example using the following procedure. The target sequences and the nucleotide location within the c-raf mRNA are given in Table XII.

15     *Cleavage Reactions:* Full-length or partially full-length, internally-labeled target RNA for ribozyme cleavage assay is prepared by *in vitro* transcription in the presence of [ $\alpha$ -<sup>32</sup>P] CTP, passed over a G 50 Sephadex column by spin chromatography and used as substrate RNA without further purification. Alternately, substrates are 5'-<sup>32</sup>P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed by pre-warming a 2X concentration of purified ribozyme in ribozyme cleavage buffer (50 mM Tris-HCl, pH 7.5 at 37°C, 10 mM MgCl<sub>2</sub>) and the cleavage reaction was initiated by adding the 2X ribozyme mix to an equal volume of substrate RNA (maximum of 1-5 nM) that was also pre-warmed in cleavage buffer. As an initial screen, assays are carried out for 1 hour at 37°C using a final concentration of either 40 nM or 1 mM ribozyme, *i.e.*, ribozyme excess. The reaction is quenched by the addition of an equal volume of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol after which the sample is heated to 95°C for 2 minutes, quick chilled and loaded onto a denaturing

polyacrylamide gel. Substrate RNA and the specific RNA cleavage products generated by ribozyme cleavage are visualized on an autoradiograph of the gel. The percentage of cleavage is determined by Phosphor Imager® quantitation of bands representing the intact substrate and the cleavage products.

5 Example 5: Ability of c-raf Ribozymes to Inhibit Smooth Muscle Cell Proliferation.

Ribozymes targeting sites in c-Raf mRNA were synthesized using modifications that confer nuclease resistance (Beigelman, 1995, *J. Biol. Chem.* 270, 25702). The ribozymes were screened for their ability to inhibit cell proliferation in serum-starved primary rat aortic smooth muscle cells as described by Jarvis et al. (1996, *RNA* 2, 419; 10 incorporated by reference herein). The ribozyme targeting site represented by Seq ID Nos 175 and 198 showed particularly high activity in inhibiting cell proliferation. An inactive control ribozyme was synthesized which had identical substrate binding arms but contained mutations in the catalytic core that eliminate cleavage activity. Inhibition of cell proliferation by active versus inactive c-Raf ribozymes is shown in Figures 37 and 15 38. The data are presented as proliferation relative to the serum-stimulated untreated control cells. Clearly the active ribozyme is showing substantial inhibition relative to both the untreated control and its corresponding inactive control, thus indicating that the inhibition of proliferation is mediated by ribozyme-mediated cleavage of c-Raf.

In several other systems, cationic lipids have been shown to enhance the 20 bioavailability of oligonucleotides to cells in culture (Bennet, C. F., et al., 1992, *Mol. Pharmacology*, 41, 1023-1033). In many of the following experiments, ribozymes were complexed with cationic lipids. The cationic lipid, Lipofectamine (a 3:1 (w/w) formulation of DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate) and dioleoyl phosphatidylethanolamine 25 (DOPE)), was purchased from Life Technologies, Inc. DMRIE (*N*-[1-(2,3-ditetradeeyloxy)propyl]-*N,N*-dimethyl-*N*-hydroxyethylammonium bromide) was obtained from VICAL. DMRIE was resuspended in CHCl<sub>3</sub> and mixed at a 1:1 molar ratio with dioleoyl phosphatidylethanolamine (DOPE). The CHCl<sub>3</sub> was evap rated, the lipid was

resuspended in water, vortexed for 1 minute and bath sonicated for 5 minutes. Ribozyme and cationic lipid mixtures were prepared in serum-free DMEM immediately prior to addition to the cells. DMEM plus additives was warmed to room temperature (about 20-25°C), cationic lipid was added to the final desired concentration and the solution was 5 vortexed briefly. RNA oligonucleotides were added to the final desired concentration and the solution was again vortexed briefly and incubated for 10 minutes at room temperature. In dose response experiments, the RNA/lipid complex was serially diluted into DMEM following the 10 minute incubation.

Serum-starved smooth muscle cells were washed twice with PBS, and the 10 RNA/lipid complex was added. The plates were incubated for 4 hours at 37°C. The medium was then removed and DMEM containing 10% FBS, additives and 10 µM bromodeoxyuridine (BrdU) was added. In some wells, FBS was omitted to determine the baseline of unstimulated proliferation.

The plates were incubated at 37°C for 20-24 hours, fixed with 0.3% H<sub>2</sub>O<sub>2</sub> in 15 100% methanol, and stained for BrdU incorporation by standard methods. In this procedure, cells that have proliferated and incorporated BrdU stain brown; non-proliferating cells are counter-stained a light purple. Both BrdU positive and BrdU negative cells were counted under the microscope. 300-600 total cells per well were counted. In the following experiments, the percentage of the total cells that have 20 incorporated BrdU (% cell proliferation) is presented. Errors represent the range of duplicate wells. Percent inhibition then is calculated from the % cell proliferation values as follows: % inhibition = 100 - 100((Ribozyme - 0% serum)/(Control - 0% serum)).

From this initial screen, hammerhead ribozyme targeted against c-raf site 1120 (Figure 36) was further tested. The active ribozyme was able to inhibit proliferation of 25 smooth muscle cell, whereas, the control inactive ribozyme, that cannot cleave c-raf RNA due to alterations in their catalytic core sequence, fails to inhibit smooth muscle cell proliferation (Figure 37). Thus, inhibition of cell proliferation by these hammerhead

sequences is due to their ability to cleave c-raf RNA, and not because of any non-ribozyme activity.

Example 6: Oligonucleotide design and preparation for cloning Defined and Random Libraries

5 The DNA oligonucleotides used in this study to construct Defined and Random Ribozyme Libraries were purchased from Life Technologies (BRL). A schematic of the oligonucleotide design used to construct said Defined or Comprehensive Ribozyme Libraries is shown in Figure 8. This example is meant to illustrate one possible means to construct such libraries. The methods described herein are not meant to be inclusive of all 10 possible methods for constructing such libraries. The oligonucleotides used to construct the hammerhead ribozyme libraries were designed as follows:

5'-CGAAATCAATTG-(N1)<sub>x</sub>-{CatalyticCore}-(N2)<sub>x</sub>-CGTACGACACGAAAGTATCG-3'

Where N1 = the Stem I target-specific binding arm of length x, Catalytic Core = the hammerhead catalytic domain 5'-CTGATGAGGCCGUUAGGCCGAAA-3', and N2 = the Stem III target specific binding arm of length x. The oligonucleotides were 15 designed to self-prime via formation of a stem-loop structure encoded at the 3' ends of the oligos (Figure 8A). This intramolecular interaction favored an unbiased extension of complex pools of ribozyme-encoding oligonucleotides. In the case of Defined Ribozyme Library described below (Figures 9-10), N1 and N2 were 8 nt each and were designed to 20 be complimentary to the RNA encoded by the purine nucleoside phosphorylase (PNP) gene. In the case of Random Ribozyme Libraries, N1 and N2 were randomized during synthesis to produce a single pool of all possible hammerhead ribozymes.

In the example shown (Figures 9-10), oligonucleotides encoding 40 different PNP-specific hammerhead ribozymes (greater than 40 ribozymes can be used) 25 were pooled to a final concentration of 1  $\mu$ M total oligonucleotides (2.5 nM each individual oligo). Oligos were heated to 68°C for 30 min and then cooled to ambient temperature to promote formation of the 3' stem-loop for self-priming (Figure 8A). The

3' stem loop was extended (Figure 8B) using Klenow DNA polymerase (1  $\mu$ M total oligonucleotides in 1 ml of 50 mM Tris pH 7.5, 10mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA, 25  $\mu$ g M dNTP mix, and 200 U Klenow) by incubating for 30 min at 37°C. The reaction mixtures were then heated to 65°C for 15 min to inactivate the polymerase. The double-stranded 5 oligos (approximately 30  $\mu$ g) were digested with the 100 U of the 5' restriction endonuclease Mfe I (NEB) as described by the manufacturer, then similarly digested with the 3' restriction endonuclease BsiWI (Figure 8C). To reduce the incidence of multiple ribozyme inserts during the cloning steps, the cleaved products were treated with Calf Intestinal Phosphatase (CIP, Boehringer Mannheim) as described by the manufacturer to 10 remove the phosphate groups at the 5' ends. This step inhibits intra- and intermolecular ligation of the ribozyme-encoding fragments. Full-length product corresponding to the double-stranded, restriction digested and phosphatase-treated products was gel-purified following electrophoresis through 10% non-denaturing acrylamide gels prior to cloning to enrich for full-length material.

15 Example 7: Cloning of Defined and Random Libraries

The cloning vectors used contained the following cloning sites: 5'- MfeI - Cla I - BsiWI -3'. Vectors were digested with Mfe I and BsiWI prior to use. Thus, vectors cleaved with both enzymes should lack the Cla I site present between the sites, while vectors cleaved with only one of the enzymes should still retain the Cla I site. Pooled 20 oligos were ligated to vector using a 2:1 or 5:1 molar ratio of double-stranded oligo to vector in 50-mL reactions containing 500 ng vector and 5 U ligase in 1x ligase buffer (Boehringer Mannheim). Ligation reactions were incubated over night at 16°C, then heated to 65°C 10 min to inactivate the ligase enzyme. The desired products contain a single ribozyme insert and lack the original Cla I site included between the Mfe I and 25 BsiWI cloning sites. Any unwanted, background vector lacking ribozyme inserts and thus still containing the Cla I sites were inactivated by cleaving the product with 5 U of the restriction endonuclease Cla I for 1 h at 37°C. Approximately 150 ng of ligated vector

was used to transform 100  $\mu$ l XL-2 Blue competent bacteria as described by the supplier (Stratagene).

Example 8: Simultaneous screening of 40 different ribozymes targeting PNP using Defined Ribozyme Libraries.

5 A Defined Ribozyme Library containing 40 different hammerhead ribozymes targeting PNP was constructed as described above (Figures 8-10). PNP is an enzyme that plays a critical role in the purine metabolic/salvage pathways. PNP was chosen as a target because cells with reduced PNP activity can be readily selected from cells with wild-type activity levels using the drug 6-thioguanosine. This agent is not toxic to cells until it is  
10 converted to 6-thioguanine by PNP. Thus, cells with reduced PNP activity are more resistant to this drug and can be selectively grown in concentrations of 6-thioguanosine that are toxic to cells with wild-type activity levels.

The PNP-targeted Defined Ribozyme Library expression vectors were converted into retroviral vector particles, and the resulting particles were used to  
15 transduce the Sup T1 human T cell line. A T-cell line was chosen for study because T lymphocytes are more dependent on the purine salvage pathway and thus are highly susceptible to 6-thioguanosine killing. Two weeks after transduction, the cells were challenged with 10 mmol 6-thioguanosine. Resistant cells began to emerge two weeks after initiation of selection. 6-Thioguanosine-resistant cells were harvested, and the  
20 ribozyme-encoding region of the expression vector was amplified using PCR and sequenced. The sequence pattern of the ribozyme region in the selected cells was significantly different from that produced from the starting library shown in Figure 9. In the original library, sequences of the binding arms were ambiguous due to the presence of all 40 PNP-targeted ribozymes (Figure 9). However, the sequence of the ribozyme-encoding regions from the 6-thioguanosine selected cells was clearly weighted towards one of the ribozymes contained in the original pool - the ribozyme designed to cleave at nucleotide #32 of PNP mRNA. These data suggests that the ribozyme targeting position  
25

32 of the PNP mRNA appears to be more active than the other 39 PNP-targeted ribozymes included in the pool.

Example 9: Optimizing Loop II sequence of a Hammerhead Ribozyme (HH-B) for Enhanced Catalytic Rates

5 To test the feasibility of the combinatorial approach described in Figure 12 approach, Applicant chose to optimize the sequence of loop-II of a hammerhead ribozyme (HH-B) (see Figure 22). Previous studies had demonstrated that a variety of chemical modifications and different sequences within loop-II may have significant effects on the rate of cleavage *in vitro*, despite the fact that this sequence is not phylogenetically 10 conserved and can in fact be deleted completely. According to the standard numbering system for the hammerhead ribozyme, the four positions within loop II are numbered I2.1, I2.2, I2.3, and I2.4. The Starting Ribozyme (HH-B) contained the sequence G<sub>I2.1</sub> A<sub>I2.2</sub> A<sub>I2.3</sub> A<sub>I2.4</sub>. For simplicity, the four positions will be numbered 5' to 3': G<sub>I2.1</sub> = 1; A<sub>I2.2</sub> = 2; A<sub>I2.3</sub> = 3; A<sub>I2.4</sub> = 4. The remainder of the hammerhead ribozyme "template" remained 15 constant and is based on a previously described hammerhead motif (Draper *et al.*, International PCT Publication No. WO 95/13380, incorporated by reference herein).

A strategy for optimizing the four (number of Classes = 4) loop-II positions is illustrated in Figure 180. The four standard ribose nucleotides (A, C, U and G) were chosen to construct the ribozyme pools (n = 4). In the first step, four different pools were 20 synthesized by the nucleotide building block mixing approach described herein. Applicant first chose to "fix" (designated F) position 3 because preliminary experiments indicated that the identity of the base at this position had the most profound effects on activity; positions 1, 2 and 4 are random. The four pools were assayed under stoichiometric conditions (1μM ribozyme; 1μM substrate), to help ensure that the entire 25 population of ribozymes in each pool was assayed. Substrate and ribozyme were pre-annealed and the reactions were initiated with the addition of 10mM MgCl<sub>2</sub>. The rate of cleavage for each library was derived from plots of fraction of substrate cleaved as a function of time. Reactions were also performed simultaneously with the starting

ribozyme (i.e., homogenous, loop-II = GAAA). The relative rate of cleavage for each library ( $k_{rel}$ ) was calculated by dividing the observed rate of the library by the rate of the control/starting ribozyme and is plotted in Figure 21. The error bars indicate the standard error derived from the curve fits. The results show that all four pools had similar rates 5 ( $k_{rel}$ ); however, the library possessing "U" at position 3 was slightly faster.

Ribozyme pools were again synthesized (Class 2) with position 3 being made constant (U<sub>3</sub>), position 4 was fixed (F<sub>4</sub>) and positions 1 and 2 were random (X). The four pools were assayed as before; the pool containing "A" at position 4 was identified as the most desirable pool. Therefore, during the synthesis of the next pool (Class 3), positions 10 3 and 4 were constant with U<sub>3</sub> and A<sub>4</sub>, position 2 was fixed (F<sub>2</sub>) and position 1 was random (X). The four pools were again assayed; all four pools showed very similar, but substantially elevated rates of cleavage. The pool containing U at position 2 was identified as the fastest. Therefore, during the synthesis of the final four ribozymes (Class 15 4), position 3, 4 and 2 were made constant with U<sub>3</sub>, A<sub>4</sub> and U<sub>2</sub>; position 1 was fixed with A, U, C or G. The final ribozyme containing G at position 4 was clearly identified as the fastest ribozyme, allowing the identification of G<sub>12.1</sub> U<sub>12.2</sub> U<sub>12.3</sub> A<sub>12.4</sub> as the optimized ribozyme motif.

To confirm that the final ribozyme (G<sub>12.1</sub> U<sub>12.2</sub> U<sub>12.3</sub> A<sub>12.4</sub>) was indeed faster than the starting ribozyme (G<sub>12.1</sub> A<sub>12.2</sub> A<sub>12.3</sub> A<sub>12.4</sub>), we compared the two ribozymes (illustrated in 20 Figure 22) under single-turnover conditions at saturating ribozyme concentrations. The observed rates should therefore measure the rate of the chemical step,  $k_2$ . The fraction of substrate remaining uncleaved as a function of time is shown in Figure 22 (lower panel), and the derived rate contents are shown. The results show that the optimized ribozyme cleaves >10 times faster (3.7 min<sup>-1</sup> vs. 0.35 min<sup>-1</sup>) than the starting ribozyme.

25 Example 10: Optimizing Core Chemistry of a Hammerhead Ribozyme (HH-A)

To further test the feasibility of the approach described in Figure 12, we chose to optimize the three pyrimidine residues within the core of a hammerhead ribozyme (HH-

A). These three positions (shown in Figure 13 as U7, U4 and C3) were chosen because previous studies indicated that these positions are critical for both stability (Beigelman *et al.*, 1995, *supra*) and activity (Ruffner *et al.*, 1990, *supra*; Burgin *et al.*, 1996, *supra*) of the ribozyme. According to the standard numbering system for the hammerhead 5 ribozyme, the three pyrimidine positions are 7, 4 and 3. For construction of the libraries, the ribozyme positions are numbered 3' to 5': position 24 = 7, position 27 = 4, and position 28 = 3 (see Figure 13). The remainder of the hammerhead ribozyme "template" remained constant and is based on a previously described hammerhead motif (Thompson *et al.*, US Patent No. 5,610,052, incorporated by reference herein). The starting ribozyme 10 template is targeted against nucleotide position 823 of k-ras mRNA (Site A). Down regulation of this message, as a result of ribozyme action, results in the inability of the cells to proliferate. Therefore in order to optimize a ribozyme, we chose to identify "variants" which were successful in inhibiting cell proliferation.

Cell Culture Assay:

15 *Ribozyme:lipid complex formation*

Ribozymes and LipofectAMINE were combined DMEM at final concentrations of 100 nM and 3.6  $\mu$ M, respectively. Complexes were allowed to form for 15 min at 37 C in the absence of serum and antibiotics.

*Proliferation Assay*

20 Primary rat aortic smooth muscle cells (RASMC) were seeded at a density of 2500 cells/well in 48 well plates. Cells were incubated overnight in DMEM, supplemented with 20% fetal bovine serum (FBS), Na-pyruvate, penicillin (50 U/ml), and streptomycin (50  $\mu$ g/ml). Subsequently cells were rendered quiescent by a 48 h incubation in DMEM with 0.5% FBS.

25 Cells were incubated for 1.5 h with serum-free DMEM ribozyme:lipid complexes. The medium was replaced and cells were incubated for 24 h in DMEM with 0.25% FCS.

Cells were then stimulated with 10% FBS for 24 h.  $^3\text{H}$ -thymidine (0.3  $\mu\text{Ci}/\text{well}$ ) was present for the last 12 h of serum stimulation.

At the end of the stimulation period the medium was aspirated and cells were fixed in icecold TCA (10%) for 15 min. The TCA solution was removed and wells were 5 washed once with water. DNA was extracted by incubation with 0.1 N NaOH at RT for 15 min. Solubilized DNA was quantitatively transferred to minivials. Plates were washed once with water. Finally,  $^3\text{H}$ -thymidine incorporation was determined by liquid scintillation counting.

A strategy for optimizing the three (number of Class = 3) pyrimidine residues is 10 illustrated in Figure 20. Ten different nucleotide analogs (illustrated in Figure 15) were chosen to construct the ribozyme library ( $n = 10$ ). In the first step, ten different pools (Class 1) were synthesized by the mix and split approach described herein. Positions 24 and 27 were random and position 28 was fixed with each of the ten different analogs. The ten different pools were formulated with a cationic lipid (Jarvis *et al.*, 1996, *RNA*, 2,419; 15 incorporated by reference herein), delivered to cells *in vitro*, and cell proliferation was subsequently assayed (see Figure 16). A positive control (active ribozyme) inhibited cell proliferation by ~50% and an inactive control (inactive) resulted in a less than 25% reduction in cell proliferation. The ten ribozyme pools resulted in intermediate levels of reduction. However, the best pool could be identified as  $X_{24} X_2, 2'\text{-MTM-U}_{28}$  (positions 20 24 and 27 random; 2'-O-MTM-U at position 28). Therefore, a second ribozyme library (Class 2) was synthesized with position 28 constant (2'-O-MTM-U); position 24 was random ( $X_{24}$ ) and position 27 was fixed with each of the ten different analogs ( $F_{27}$ ). Again, the ten pools were assayed for their ability to inhibit cell proliferation. Among 25 Class 2, two pools inhibited proliferation equally well:  $X_{24} 2'\text{-C-allyl-U}_2, 2'\text{-O-MTM-U}_{28}$  and  $X_{24} 2'\text{-O-MTM-C}_2, 2'\text{-O-MTM-U}_{28}$ . Because a single "winner" could not be identified in Class 2, position 27 was made constant with either 2'-C-allyl-U or with 2'-O-MTM-C and the ten analogs were placed individually at position 24 (Class 3). Therefore in Class 3, twenty different ribozymes were assayed for their ability to inhibit

cell proliferation. Because both positions 27 and 28 are constant, the final twenty ribozymes contain no random positions. Thus in the final group (Class 3), pure ribozymes and not pools were assayed. Among the final groups four ribozymes inhibited cell proliferation to a greater extent than the control ribozyme (Figure 22). These four 5 winners are illustrated in Figure 23A. Figure 23B shows general formula for four different motifs. A formula for a novel ribozyme motif is shown in Figure 18.

Example 11: Identifying Accessible Sites for Ribozyme Action in a target

In the previous two examples (9 and 10), positions within the catalytic domain of the hammerhead ribozyme were optimized. The number of groups that needed to be 10 tested equals = the total number of positions within the ribozyme that were chosen to be tested. A similar procedure can be used on the binding arms of the ribozyme. The sequence of the binding arms determines the site of action of the ribozyme. The combinatorial approach can be used to identify those sites by essentially testing all possible arm sequences. The difficulty with this approach is that ribozymes require a 15 certain number of base pairs (12-16) in order bind tightly and specifically. According to the procedure outlined above, this would require 12-16 different groups of ribozyme pools; 12-16 positions would have to be optimized which would require 12-16 different groups being synthesized and tested. Each pool would contain the four different nucleotides (A, C, U and G) or nucleotide analogs ( $n = 4$ ). It would be very time 20 consuming to test each group, identify the best pool, synthesize another group of ribozyme pools with one additional position constant, and then repeat the procedure until all 12-16 groups had been tested. However it is possible to decrease the number of groups by testing multiple positions within a single group. In this case, the number of groups within a group equals the number of nucleotides or analogs in the random mixture 25 (i.e.,  $n$ ) to the  $w$  power, where  $w$  equals the number of positions fixed in each group. The number of groups that need to be synthesized to optimize the final ribozyme equals the total number of positions to be optimized divided by the number of positions ( $w$ ) tested

within each group. The number of pools in each group =  $n^w$ . The number of groups = total number of positions / w.

For example, Figure 23 illustrates this concept on a hammerhead ribozyme containing 12 base pair binding arms. Each of the two binding arms form 6 base pairs with its corresponding RNA target. It is important to note that for the hammerhead ribozyme one residue (A15.1) must remain constant; A15.1 forms a base pair with a substrate nucleotide (U16.1) but is also absolutely required for ribozyme activity. It is the only residue within the hammerhead ribozyme that is part of both the catalytic domain, and the binding domain (arms). In the example this position is not optimized. In the first 5 Group, three positions are fixed (designated F) with the four different 2'-O-methyl nucleotides (A, C, U and G). The 2'-O-methyl modification stabilizes the ribozyme against nuclease degradation and increases the binding affinity with its substrate. The total number of pools in each group does not equal n, as in the previous examples. The number of pools in each group equals  $4^3$  = (four analogs)<sup>(number of positions fixed; 3)</sup> = 10 64. In all 64 pools, all other positions in the arm are made random (designated X) by the nucleotide mixing building block approach. The catalytic domain is not considered in 15 this example and therefore remains part of the ribozyme template (i.e., constant).

In the first step, all 64 ribozyme pools are tested. This test may be cleavage *in* 20 *vitro* (see Example 9), or efficacy in a cellular (see Example 10) or animal model, or any other assayable end-point. This end-point however, should be specific to a particular RNA target. For example, if one wishes to identify accessible sites within the mRNA of GeneB, a suitable end-point would be to look for decreased levels of GeneB mRNA after ribozyme treatment. After a winning pool is identified, since each pool specifies the identity of three positions (w), three positions can be made constant for the next group 25 (Class 2). Class 2 is synthesized containing 64 different pools; three positions that were fixed in Class 1 are now constant (designated Z), three more positions are fixed (F), and the remaining positions (X) are a random mix of the four nucleotides. The 64 pools are assayed as before, a winning pool is identified, allowing three more positions to be

constant in the next Class of ribozyme pools (Class 3) and the process is repeated again. In the final Class of ribozymes (Class 4), only two positions are fixed, all other positions have been previously fixed. The total number of ribozymes is therefore  $n^w = 4^2 = 16$ ; these ribozymes also contain no random positions. In the final step (step 4), the 16 5 ribozymes are tested; the winning ribozyme defines the sequence of the binding arms for a particular target.

Fixing multiple positions within a single group it is possible to decrease the overall number of groups that need to be tested. As mentioned, this is particularly useful when a large number of different positions need to be optimized. A second advantage to 10 this approach is that it decreases the complexity of molecules in each pool. If one would expect that many combinations within a given pool will be inactive, by decreasing the number of different ribozymes in each pool, it will be easier to identify the "winning" pool. In this approach, a larger number of pools have to be tested in each group, however, the number of groups is smaller and the complexity of each ribozyme pool is smaller. 15 Finally, it should be emphasized there is not a restriction on the number of positions or analogs that can be tested. There is also no restriction on how many positions are tested in each group.

#### Example 12: Identifying new RNA targets for Ribozymes

As described above for identifying ribozyme-accessible sites, the assay used to 20 identify the "winning" pool of ribozymes is not defined and may be cleavage *in vitro* (see Example 8), or efficacy in a cellular (see Example 9) or animal model, or any other assayable end-point. For identifying accessible sites, this end-point should be specific to a particular RNA target (e.g., mRNA levels). However, the end-point could also be nonspecific. For example, one could choose a disease model and simply identify the 25 winning ribozyme pool based on the ability to provide a desired effect. In this case, it is not even necessary to know what the cellular target that is being acted upon by the ribozyme is. One can simply identify a ribozyme that has a desired effect. The advantage to this approach is that the sequence of the binding arms will be complementary to the

RNA target. It is therefore possible to identify gene products that are involved in a disease process or any other assayable phenotype. One does not have to know what the target is prior to starting the study. The process of identifying an optimized ribozyme (arm combinatorial) identifies both the drug (ribozyme) and the RNA target, which may 5 be a known RNA sequence or a novel sequence leading to the discovery of new genes.

Example 13: Identifying New Ribozyme Catalytic Domains

In the previous two examples, positions within the binding domain of the hammerhead ribozyme were varied and positions within the catalytic domain were not changed. Conversely, it is possible to vary positions within the catalytic domain, without 10 changing positions within the binding arms, in order to identify new catalytic motifs. An example is illustrated in Figure 24. The hammerhead ribozyme, for example comprises about 23 residues within the catalytic domain. It is unclear how many of these 23 positions are required to obtain a functional catalytic domain, however it is reasonable to presume that if a large number of functionally diverse nucleotide analogs can be used to 15 construct the pools, a relatively small number of positions could constitute a functional catalytic domain. This may especially be true if analogs are chosen that one would expect to participate in catalysis (e.g., acid/base catalysts, metal binding, etc.). In the example illustrated in Figure 24, four positions (designated 1, 2, 3 and 4) are chosen. In the first step, ribozyme libraries (Class 1) are constructed: position 1 is fixed ( $F_1$ ) and positions 2, 20 3 and 4 are random ( $X_2$ ,  $X_3$  and  $X_4$ , respectively). In step 2, the pools (the number of pools tested depends on the number of analogs used;  $n$ ) are assayed for activity. This testing may be performed *in vitro* or in a cellular or animal model. Whatever assay that is used, the pool with the most activity is identified and libraries (class 2) are again synthesized with position 1 now constant ( $Z_1$ ) with the analog that was identified in class 25 1. In class 2, position 2 is fixed ( $F_2$ ) and positions 3 and 4 are random ( $X_3$  and  $X_4$ ). This process is repeated until every position has been made constant, thus identifying the catalytic domain or a new motif.

EXAMPLE 14: Determination of Coupling Efficiency of the phosphoramidite derivatives of 2'-C-allyl-uridine, 1; 4'-thio-cytidine, 2; 2'-methylthiomethyl-uridine, 3; 2'-methylthiomethyl-cytidine, 4; 2'-amino-uridine, 5; N3-methyl-uridine, 6; 1-b-D-(ribofuranosyl)-pyridin-4-one, 7; 1-b-D-(ribofuranosyl)-pyridin-2-one, 8; 1-b-D-(ribofuranosyl)-phenyl, 9; 6-methyl-uridine, 10 to be used in a split and mix approach.

The determination of the coupling efficiency of amidites 1 to 10 was assessed using ten model sequences agacXGAuGa (where upper case represents ribonucleotide residues, lower case represents 2'-O-methyl ribonucleotide residues and X is amidites 1 to 10, to be used in the construction of a hammerhead ribozyme library wherein the 10 modified amidites 1 to 10 would be incorporated. Ten model sequences were synthesized using ten 0.112 g aliquots of 5'-O-DMT-2'-O-Me-Adenosine Polystyrene (PS) solid-support loaded at 22.3  $\mu$ mol/g and equivalent to a 2.5  $\mu$ mol scale synthesis. Synthesis of these ten decamers were performed on ABI 394 DNA synthesizer (Applied Biosystems, Foster City, Calif.) using standard nucleic acid synthesis reagents and synthesis protocols, 15 with the exception of an extended (7.5 min) coupling time for the ribonucleoside phosphoramidites and phosphoramidites 1, 2, 3, 4, 6, 7, 8, 9, 10, 12.5 min coupling time for the 2'-amino-uridine phosphoramidite, amidite 5 and 2.5 min coupling time for the 2'-O-methyl nucleoside phosphoramidites.

Oligomers were cleaved from the solid support by treatment with a 3:1 mixture of 20 ammonium hydroxide:absolute ethanol at 65 degree C for 4 hrs followed by a desilylation treatment and butanol precipitation as described in Wincott et al. (Wincott et al, *Nucleic Acids Res*, 1995, 23, 2677-2684; incorporated by reference herein). Oligonucleotides were analyzed directly on an anion-exchange HPLC column (Dionex, Nucleopac, PA-100, 4x250 mm) using a gradient of 50% to 80% of B over 12 minutes (A = 10 mM sodium perchlorate, 1 mM Tris, pH 9.43; B = 300 mM sodium perchlorate, 1 mM Tris, pH 9.36) and a Hewlett-Packard 1090 HPLC system.

The average stepwise yield (ASWY), indicating the coupling efficiency of phosphoramidites, 1 to 10, were calculated from peak-area percentages according to the

equation  $ASWY = (FLP\%)^{1/n}$  where  $FLP\%$  is the percentage full-length product in the crude chromatogram and  $n$  the number of synthesis cycles.  $ASWY$  ranging from of 96.5% to 97.5% were obtained for phosphoramidites, 1 to 10. The experimental coupling efficiencies of the phosphoramidites 1 to 10, as determined using a standard 5 spectrophotometric dimethoxytrityl assay were in complete agreement with the  $ASWY$  and were judged satisfactory to proceed with the X24, X27, X28 ribozyme library synthesis.

10 EXAMPLE 15: Determination of optimal relative concentration of a mixture of 2'-O-methyl-guanosine, cytidine, uridine and adenosine providing equal representation of the four nucleotides.

A mixture N, composed of an equimolar mixture of the four 2'-O-Me- nucleoside phosphoramidites (mG=2'-O-methyl guanosine; mA=2'-O-methyl adenosine; mC=2'-O-methyl cytidine; mU=2'-O-methyl uridine) was used in the synthesis of a model sequence TTXXXXTTB, where T is 2'-deoxy-thymidine and B is a 2'-deoxy-inverted abasic 15 polystyrene solid-support as described in Example 14. After standard deprotection (Wincott *et al.*, *supra*), the crude nonamer was analyzed on an anion-exchange HPLC column (see example 6). From the HPLC analysis, an averaged stepwise yield ( $ASWY$ ) of 99.3% was calculated (see example 14) indicating that the overall coupling efficiency of the mixture N was comparable to that of 2'-deoxythymidine. To further assess the 20 relative incorporation of each of the components within the mixture, N, the full-length product TTXXXXTTB (over 94.3% at the crude stage) was further purified and subjected to base composition analysis as described herein. Purification of the FLP from the failures is desired to get accurate base composition.

**Base composition analysis summary:**

25 A standard digestion/HPLC analysis was performed: To a dried sample containing 0.5 A.sub.260 units of TTXXXXTTB, 50  $\mu$ l mixture, containing 1 mg of nuclease P1 (550 units/mg), 2.85 ml of 30 mM sodium acetate and 0.3 ml of 20 mM aqueous zinc chloride, was added. The reaction mixture was incubated at 50 degrees C overnight.

Next, 50  $\mu$ l of a mixture comprising 500  $\mu$ l of alkaline phosphatase (1 units/ $\mu$ l), 312  $\mu$ l of 500 mM Tris pH 7.5 and 2316  $\mu$ l water was added to the reaction mixture and incubated at 37 degrees C for 4 hours. After incubation, the samples were centrifuged to remove sediments and the supernatant was analyzed by HPLC on a reversed-phase C18 column 5 equilibrated with 25 mM KH<sub>2</sub>PO<sub>4</sub>. Samples were analyzed with a 5% acetonitrile isocratic gradient for 8 min followed by a 5% to 70% acetonitrile gradient over 8 min.

The HPLC percentage areas of the different nucleoside peaks, once corrected for the extinction coefficient of the individual nucleosides, are directly proportional to their molar ratios.

10 The results of these couplings are shown in Table IV.

Nucleoside	dT 0.1 M	2'-OMe-C 0.025M	2'-OMe-U 0.025M	2'-OMe-G 0.025M	2'-OMe-A 0.025M
% area	43.81	6.04	14.07	18.54	17.54
Epsilon 260 nm	8800	7400	10100	11800	14900
moles	0.00498	0.00082	0.00139	0.00157	0.00118
equivalent	4	0.656	1.119	1.262	0.946

As can be seen in Table IV, the use of an equimolar mixture of the four 2'-O-methyl phosphoramidites does not provide an equal incorporation of all four amidites, but favors 2'-O-methyl-U and G and incorporates 2'-O-methyl-A and C to a lower efficiency.

15 To alleviate this, the relative concentrations of 2'-O-methyl-A, G, U and C amidite were adjusted using the inverse of the relative incorporation as a guide line. After several iterations, the optimized mixture providing nearly identical incorporation of all four amidites was obtained as shown in Table V below. The relative representation do not exceed 12% difference between the most and least incorporated residue corresponding to 20 a +/- 6% deviation from equimolar incorporation.

Nucleoside	dT 0.1M	2'-OMe-C 0.032M	2'-OMe-U 0.022M	2'-OMe-G 0.019M	2'-OMe-A 0.027M
% area	44.47	8.91	11.81	15.53	19.28
Epsilon 260 nm	8800	7400	10100	11800	14900
moles	0.00505	0.00120	0.00117	0.00132	0.00129
equivalent	4	0.953	0.926	1.042	1.024

EXAMPLE 16: A Non-competitive coupling method for the preparation of the X24, X27 and N28 ribozyme library 5'- a<sub>1</sub>c<sub>2</sub>a<sub>3</sub>a<sub>4</sub>ag aFX GAX Gag gcg aaa gcc Gaa Agc ccu cB -3'  
wherein 2'-C-allyl-uridine, 1; 4'-thio-cytidine, 2; 2'-methylthiomethyl-uridine, 3; 2'-  
5 methylthiomethyl-cytidine, 4; 2'-amino-uridine, 5; N3-methyl-uridine, 6; 1-b-D-  
(ribofuranosyl)-pyrimidine-4-one, 7; 1-b-D-(ribofuranosyl)-pyrimidine-2-one, 8; 1-b-D-  
(ribofuranosyl)-phenyl, 9; and/or 6-methyl-uridine, 10 are incorporated at the X24, X27  
and F28 positions through the mix and split approach.

The synthesis of ten different batches of 2.5  $\mu$ mol scale Gag gcg aaa gcc Gaa Agc  
10 ccu cB sequence was performed on 2'-deoxy inverted abasic polystyrene solid support B  
on a 394 ABI DNA synthesizer (Applied Biosystems, Foster City, CA). These ten  
aliquots were then separately reacted with phosphoramidite building blocks 1 to 10  
according to the conditions described in example 11. After completion of the individual  
incorporation of amidites 1 to 10, their coupling efficiencies were determined to be above  
15 95 % as judged by trityl monitoring. The 10 different aliquots bearing the ten different  
sequences were mixed thoroughly and divided into ten equal subsets. Each of these  
aliquots were then successively reacted with ribo-A, ribo-G amidites and one of the  
amidites 1 to 10. The ten aliquots were combined, mixed and split again in 10 subsets.  
At that point, the 10 different polystyrene aliquots, exhibiting the following sequence: X  
20 GAX Gag gcg aaa gcc Gaa Agc ccu cB, were reacted again with amidites 1 to 10  
separately. The aliquots were not mixed, but kept separate to obtain a unique residue at

the 28th position of each of the ten pools. The ribozyme synthesis was then finished independently to yield ten random ribozymes pools. Each pool comprises a 3'-terminal inverted abasic residue **B**, followed by the sequence Gag gcg aaa gcc Gaa Agc ccu c, followed with one random position *X* in the 24th position corresponding to a mixture of 5 amidites 1 to 10, followed by the sequence GA, followed one random position *X* in the 27th position corresponding to a mixture of amidites 1 to 10, followed by a fixed monomer **F** (one of the amidites 1 to 10) in the 28th position and finally the 5'-terminal sequence a<sub>s</sub>c<sub>s</sub>a<sub>s</sub>a<sub>s</sub>a g a. This is represented by the sequence notation 5'- a<sub>s</sub>c<sub>s</sub>a<sub>s</sub>a<sub>s</sub>ag aFX GAX Gag gcg aaa gcc Gaa Agc ccu cB-3', in which *X* are random positions and **F** is a unique fixed position. The total complexity of such a ribozyme library was 10<sup>3</sup> or 1,000 members separated in 10 pools of 100 different ribozyme sequences each.

EXAMPLE 17: Competitive coupling method (monomer mixing approach) for the preparation of the x<sub>2-6</sub> and X<sub>10-35</sub>, "binding arms" ribozyme library

Synthesis of 5'-x<sub>s</sub>x<sub>s</sub>x xFF cuG Au G Agg ccg uua ggc cGA AAF xxx xB-3' is described, with F being a defined 2'-*O*-methyl-ribonucleoside chosen among 2'-*O*-methyl-ribo-adenosine (mA), -guanosine (mG), -cytidine (mC), -uridine (mU) and x being an equal mixture of 2'-*O*-methyl-ribo-adenosine, -guanosine, -cytidine, -uridine.

The syntheses of this ribozyme library was performed with an ABI 394 DNA synthesizer (Applied Biosystems, Foster City, Calif.) using standard nucleic acid synthesis reagents and synthesis protocols, with the exception of an extended (7.5 min) coupling time for the ribonucleoside phosphoramidites (upper case) and 2'-amino-uridine phosphoramidite, u, (2.5 min) coupling time for the 2'-*O*-methyl-ribonucleoside phosphoramidites (lower case) and the 2'-*O*-methyl-ribonucleoside phosphoramidites mixture, n.

25 Sixty four (64) batches of 0.086 g aliquots of 3'-*O*-DMT-2'-deoxy-inverted abasic-Polystyrene (**B**) solid-support loaded at 29  $\mu$ mol/g and equivalent to a 2.5  $\mu$ mol

scale synthesis were individually reacted with a 27:32:19:22 / v:v:v:v mixture, x, of mA:mC:mG:mU diluted in dry acetonitrile to 0.1 M as described in example 7. This synthesis cycle was repeated for a total of four times. The 64 aliquots were then grouped into four subsets of sixteen aliquots (Class 1) that were reacted with either mA, mG, mC, 5 mU to synthesize the n6 position. This accomplished, the sequence: 5'- cuG Au G Agg ccg uua ggc cGA AA was added onto the 6 position of the 64 aliquots constituting Class 1. Each subset of Class 1 was then divided into four subsets of four aliquots (Class 2) that were reacted with either mA, mG, mC, mU to synthesize the F30 position. Each subset of Class 2 was then divided into four subsets of one aliquot (Class 3) that were reacted with 10 either mA, mG, mC, mU to synthesize the F31 position. Finally, the random sequence 5'- x<sub>s</sub>x<sub>s</sub>x x was added onto each of the 64 aliquots.

The ribozyme library yielded sixty four random ribozymes pools each having an equal mixture of the four 2'-O-methyl-nucleoside at the position x2 to 6 and x30 to 35, and a defined 2'-O-methyl-nucleoside chosen among mA, mC, mG, mU at the positions 15 F6, F30 and F31. The total complexity of such a "binding arms" ribozyme library was 4<sup>11</sup> or 4,194,304 members separated in 64 pools of 65,536 different ribozyme sequences each.

EXAMPLE 18: Competitive coupling method (monomer mixing approach) for the preparation of the position 15 to 18 "loop II" ribozyme library

20 **Synthesis of 5' UCU CCA UCU GAU GAG GCC XXF XGG CCG AAA AUC CCU 3'** is described, with F being a defined ribonucleoside chosen among adenosine (A), guanosine (G), cytidine (C), uridine (U) and X being an equal mixture of adenosine (A), guanosine (G), cytidine (C), uridine (U).

25 The syntheses of this ribozyme library was performed with an ABI 394 DNA synthesizer (Applied Biosystems, Foster City, Calif.) using standard nucleic acid synthesis reagents and synthesis protocols, with the exception of an extended (7.5 min)

coupling time for the ribonucleoside phosphoramidites (A, G, C, U) and the ribonucleoside phosphoramidite mixture, X.

Four batches (4) of 2.5  $\mu$ mol scale of GG CCG AAA AUC CCU sequence were synthesized on 0.085 g samples of 5'-O-DMT-2'-O-TBDMS-3'-succinyl-uridine-5 Polystyrene (U) solid-support loaded at 29.8  $\mu$ mol/g. To synthesize the position X15, the four aliquots of solid-supports were individually reacted with a 30:26:24:20 / v:v:v:v mixture, X, of A:C:G:U diluted in dry acetonitrile to 0.1 M according to the optimized conditions for the DNA phosphoramidites mixed-base coupling as described in the DNA Synthesis Course Manual published by Perkin-Elmer-Applied Biosystem Division. 10 (DNA Synthesis Course Manual : Evaluating and isolating synthetic oligonucleotides, the complete guide, p. 2-4, Alex Andrus, August 1995). The four aliquots of solid-supports were then individually reacted with either of the four ribonucleoside phosphoramidites (A, G, C, U) to create the F16 position. The position X17 and X18 were then added onto the F16 (either A, G, C or U) of the four aliquots of solid-supports by repeating twice the 15 same procedure used for the position X15.

The synthesis of the ribozyme library was then ended by adding the sequence 5'-UCU CCA UCU GAU GAG GCC on the position X18 of each of the four subsets of the ribozyme library. The ribozyme library yielded four random ribozymes pools that each have an equal mixture of the four ribonucleoside (A, G, C and U) at the position X15, 20 X17 and X18, and a discrete ribonucleoside chosen among A, C, G or U at the positions F16. The total complexity of such a loop II ribozyme library was 256 members separated in 4 pools of 64 different ribozyme sequences.

Example 19: Arm-Combinatorial Library Screening For Bcl-2, K-ras and Urokinase plasminogen Activator (UPA)

25 *Substrate synthesis through in vitro transcription:* Run-off transcripts for Bcl-2 and Kras were prepared using linearized plasmids (975 and 796 nucleotides respectively). Transcripts for UPA were produced from a PCR generated DNA fragment containing a T7 promoter (400 nucleotides). Transcription was performed using the T7 Megascript

transcription kit (Ambion, Inc.) with the following conditions: a 50ul reaction volume containing 7.5mM each of ATP, CTP, UTP, and GTP, 2mM guanosine, 5ul 10x T7 reaction buffer, 5ul T7 enzyme mix, and 0.5ug of linearized plasmid or PCR'd DNA template. The mixture was incubated at 37°C for 4 hours (6 hours for transcripts < 500 bases). Guanosine was added to the transcription reactions so that the final transcript could be efficiently 5'-end labeled without prior phosphatase treatment. Transcription volume was then increased to 200ul with buffer containing 50mM TRIS pH 7.5, 100mM KCl, and 2mM MgCl<sub>2</sub>, and spin column purified over Bio-Gel P-60 (BioRad) equilibrated in the same buffer. 100ul of transcript was then applied to 750ul of packed resin. Spin 5 column flow-through was used directly in a 5'-end labeling reaction as follows (100ul final volume): 82ul of P-60 spin column purified transcript, 10ul 10x polynucleotide kinase buffer, 4ul 10U/ul Polynucleotide Kinase (Boehringer/Mannheim) and 4ul 150uCi/ul Gamma-32P-ATP (NEN) were incubated together at 37°C for one hour. The 10 reaction volume was increased to 200ul with buffer containing 50mM TRIS pH 7.5, 100mM KCl and 2mM MgCl<sub>2</sub>, and the sample was then purified over Bio-Gel P-60 packed spin column as described above. Approximate specific activities of the 5'-end 15 labeled transcripts were determined via BioScan and stored frozen at -20°C.

*Synthesis of Ribozyme pools:*

*In vitro ribozyme-transcript cleavage reactions:* Cleavage reactions were carried out as 20 follows: 5'-end labeled transcript (~2-4 x 10<sup>4</sup> dpm/ul final) was incubated with 10uM ribozyme pool in 50mM TRIS pH 7.5, 50mM NaCl, 2mM MgCl<sub>2</sub>, and 0.01% SDS for 24-48 hours at room temperature (~22°C). An equal volume of gel loading dye (95% formamide, 0.01M EDTA, 0.0375% bromophenol blue, and 0.0375% xylene cyanol) was added to stop the reaction and the samples are heated to 95°C. Reactions (1-2 x 10<sup>5</sup> dpm 25 per lane) were run on a 5% denaturing polyacrylamide gel containing 7M urea and 1x TBE. Gels are dried and imaged using the PhosphorImager system (Molecular Dynamics). Ambion, Inc. RNA Century Marker Plus RNA standards body labeled in a T7 Megascript reaction as described above using 3ul of 10mCi/ml Alpha-<sup>32</sup>P-ATP (BioRad) and 0.5ug Century RNA template and subsequently spin column purified over

Bio-Gel P-6 (Bio-Rad) were used as a size reference on the gel. Cleavage product sizes were determined using the RNA standards which provided an approximate site of cleavage (est. Size in Figure). Because each of the ribozyme pools has three positions within the binding arms fixed, it is possible to identify all of the potential ribozyme sites

5 that can potentially be cleaved by that pool. The estimated size of the cleavage product is therefore compared with the potential sites to identify the exact site of cleavage.

This protocol has been completed on three different transcripts: Bcl-2 (figure 25), Kras (figure 26), and UPA (figure 27). The data is summarized in the figures. All potential hammerhead ribozyme cleavage sites are indicated in the graph with a short vertical line.

10 The actual sites identified are indicated in the graph. The size of the bar reflects the intensity of the cleavage product from the cleavage reaction. The combinatorial pool used to identify each site, the actual sequence of each site, the position of cleavage within the transcript (based on the known sequence), and the estimated size of the cleavage product (based on gel analysis) are listed.

15 Example 20: Reduction of Bcl-2 mRNA using Optimized Ribozymes

Two ribozymes targeted against the same site in the bcl-2 transcript (Seq.ID#9, figure 25) were synthesized, but the two ribozymes were stabilized using two different chemistries (U4/U7 amino and U4 c-allyl). Ribozymes (200 nM) were delivered using lipofectamine (7.2 mM) for 3 hours into MCF-7 cells (50% confluency). Cellular RNA

20 was harvested 24 hours after delivery, analyzed by RNase protection analysis (RPA) and normalized to GAPDH mRNA in triplicate samples. Both ribozymes gave a reduction in bcl-2 mRNA (see Figure 28). A ribozyme targeted against an irrelevant mRNA (c-myb) had no effect on the ratio of bcl-2 mRNA to GAPDH mRNA. All reduction of bcl-2 RNA was statistically significant with respect to untreated samples and

25 samples treated with the irrelevant ribozyme.

Example 21: Synthesis of purine nucleoside triphosphates: 2'-O-methyl-guanosine-5'-triphosphate

2'-O-methyl guanosine nucleoside (0.25 grams, 0.84 mmol) was dissolved in triethyl phosphate (5.0) ml by heating to 100 C for 5 minutes. The resulting clear,

colorless solution was cooled to 0 C using an ice bath under an argon atmosphere. Phosphorous oxychloride (1.8 eq., 0.141 ml) was then added to the reaction mixture with vigorous stirring. The reaction was monitored by HPLC, using a sodium perchlorate gradient. After 5 hours at 0 C, tributylamine (0.65 ml) was added followed by the 5 addition of anhydrous acetonitrile (10.0 ml), and after 5 minutes (reequilibration to 0 C) tributylammonium pyrophosphate (4.0 eq., 1.53 g) was added. The reaction mixture was quenched with 20 ml of 2M TEAB after 15 minutes at 0 C (HPLC analysis with above conditions showed consumption of monophosphate at 10 minutes) then stirred overnight at room temperature, the mixture was evaporated *in vacuo* with methanol co-evaporation 10 (4x) then diluted in 50 ml 0.05M TEAB. DEAE sephadex purification was used with a gradient of 0.05 to 0.6 M TEAB to obtain pure triphosphate (0.52 g, 66.0% yield) (elutes around 0.3M TEAB); the purity was confirmed by HPLC and NMR analysis.

Example 22: Synthesis of Pyrimidine nucleoside triphosphates: 2'-O-methylthiomethyl-uridine-5'-triphosphate

15 2'-O-methylthiomethyl uridine nucleoside (0.27 grams, 1.0 mmol) was dissolved in triethyl phosphate (5.0 ml). The resulting clear, colorless solution was cooled to 0 C with an ice bath under an argon atmosphere. Phosphorus oxychloride (2.0 eq., 0.190 ml) was then added to the reaction mixture with vigorous stirring. Dimethylaminopyridine (DMAP, 0.2eq., 25 mg) was added, the solution warmed to room temperature and the 20 reaction was monitored by HPLC, using a sodium perchlorate gradient. After 5 hours at 20 C, tributylamine (1.0 ml) was added followed by anhydrous acetonitrile (10.0 ml), and after 5 minutes tributylammonium pyrophosphate (4.0 eq., 1.8 g) was added. The reaction mixture was quenched with 20 ml of 2M TEAB after 15 minutes at 20 C (HPLC analysis with above conditions showed consumption of monophosphate at 10 minutes) 25 then stirred overnight at room temperature. The mixture was evaporated *in vacuo* with methanol co-evaporation (4x) then diluted in 50 ml 0.05M TEAB. DEAE fast flow Sepharose purification with a gradient of 0.05 to 1.0 M TEAB was used to obtain pure triphosphate (0.40 g, 44% yield) (elutes around 0.3M TEAB) as determined by HPLC and NMR analysis.

Example 23: Utilization of DMAP in Uridine 5'-Triphosphate Synthesis

The reactions were performed on 20 mg aliquots of nucleoside dissolved in 1 ml of triethyl phosphate and 19  $\mu$ l of phosphorus oxychloride. The reactions were monitored at 40 minute intervals automatically by HPLC to generate yield-of-product curves at times up to 18 hours. A reverse phase column and ammonium acetate/ sodium acetate buffer system (50mM & 100mM respectively at pH 4.2) was used to separate the 5', 3', 2' monophosphates (the monophosphates elute in that order) from the 5'-triphosphate and the starting nucleoside. The data is shown in table VI. These conditions doubled the product yield and resulted in a 10-fold improvement in the reaction time to maximum yield (1200 minutes down to 120 minutes for a 90% yield). Selectivity for 5'-monophosphorylation was observed for all reactions. Subsequent triphosphorylation occurred in nearly quantitative yield.

Materials Used in Bacteriophage T7 RNA Polymerase Reactions

BUFFER 1: Reagents are mixed together to form a 10X stock solution of buffer 1 (400 mM Tris-Cl (pH 8.1), 200 mM MgCl<sub>2</sub>, 100 mM DTT, 50 mM spermidine, and 0.1% triton X-100. Prior to initiation of the polymerase reaction methanol, LiCl is added and the buffer is diluted such that the final reaction conditions for condition 1 consisted of : 40mM tris pH (8.1), 20mM MgCl<sub>2</sub>, 10 mM DTT, 5 mM spermidine, 0.01% triton X-100, 10% methanol, and 1 mM LiCl.

BUFFER 2: Reagents are mixed together to form a 10X stock solution of buffer 2(400 mM Tris-Cl (pH 8.1), 200 mM MgCl<sub>2</sub>, 100 mM DTT, 50 mM spermidine, and 0.1% triton X-100. Prior to initiation of the polymerase reaction PEG, LiCl is added and the buffer is diluted such that the final reaction conditions for buffer 2 consisted of : 40mM tris pH (8.1), 20mM MgCl<sub>2</sub>, 10 mM DTT, 5 mM spermidine, 0.01% triton X-100, 4% PEG, and 1 mM LiCl.

BUFFER 3: Reagents are mixed together to form a 10X stock solution of buffer 3 (400 mM Tris-Cl (pH 8.0), 120 mM MgCl<sub>2</sub>, 50 mM DTT, 10 mM spermidine and 0.02% triton X-100. Prior to initiation of the polymerase reaction PEG is added and the buffer is

diluted such that the final reaction conditions for buffer 3 consisted of : 40mM tris pH (8.0), 12 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM spermidine, 0.002% triton X-100, and 4% PEG.

BUFFER 4: Reagents are mixed together to form a 10X stock solution of buffer 4 (400 mM Tris-Cl (pH 8.0), 120 mM MgCl<sub>2</sub>, 50 mM DTT, 10 mM spermidine and 0.02% triton

5 X-100. Prior to initiation of the polymerase reaction PEG, methanol is added and the buffer is diluted such that the final reaction conditions for buffer 4 consisted of : 40mM tris pH (8.0), 12 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM spermidine, 0.002% triton X-100, 10% methanol, and 4% PEG.

BUFFER 5: Reagents are mixed together to form a 10X stock solution of buffer 5 (400 10 mM Tris-Cl (pH 8.0), 120 mM MgCl<sub>2</sub>, 50 mM DTT, 10 mM spermidine and 0.02% triton X-100. Prior to initiation of the polymerase reaction PEG, LiCl is added and the buffer is diluted such that the final reaction conditions for buffer 5 consisted of : 40mM tris pH (8.0), 12 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM spermidine, 0.002% triton X-100, 1 mM LiCl and 4% PEG.

15 BUFFER 6: Reagents are mixed together to form a 10X stock solution of buffer 6 (400 mM Tris-Cl (pH 8.0), 120 mM MgCl<sub>2</sub>, 50 mM DTT, 10 mM spermidine and 0.02% triton X-100. Prior to initiation of the polymerase reaction PEG, methanol is added and the buffer is diluted such that the final reaction conditions for buffer 6 consisted of : 40mM tris pH (8.0), 12 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM spermidine, 0.002% triton X-100, 10% 20 methanol, and 4% PEG.

Example 24: Screening of Modified Nucleoside triphosphates with Mutant T7 RNA Polymerase

Each modified nucleotide triphosphate was individually tested in buffers 1 through 6 at two different temperatures (25 and 37°C). Buffers 1-6 tested at 25°C were designated 25 conditions 1-6 and buffers 1-6 test at 37°C were designated conditions 7-12 (table VII). In each condition, Y639F mutant T7 polymerase (Sousa and Padilla, *Supra*) (0.3-2 mg/20 ml reaction), NTP's (2 mM each), DNA template (10 pmol), inorganic pyrophosphatase (5U/ml) and  $\alpha$ -<sup>32</sup>P NTP(0.8 mCi/pmol template) were combined and heated at the designated temperatures for 1-2 hours. The radiolabeled NTP used was different from the

modified triphosphate being testing. The samples were resolved by polyacrylamide gel electrophoresis. Using a phosphorImager (Molecular Dynamics, Sunnyvale, CA), the amount of full-length transcript was quantified and compared with an all-RNA control reaction. The data is presented in Table VIII; results in each reaction is expressed as a 5 percent compared to the all-ribonucleotide triphosphate (rNTP) control. The control was run with the mutant T7 polymerase using commercially available polymerase buffer (Boehringer Mannheim, Indianapolis, IN).

Example 25: Incorporation of Modified NTP's using Wild-type T7 RNA polymerase

Bacteriophage T7 RNA polymerase was purchased from Boehringer Mannheim at 10 0.4 U/ $\mu$ L concentration. Applicant used the commercial buffer supplied with the enzyme and 0.2  $\mu$ Ci alpha- $^{32}$ P NTP in a 50  $\mu$ L reaction with nucleotides triphosphates at 2 mM each. The template was double-stranded PCR fragment, which was used in previous screens. Reactions were carried out at 37°C for 1 hour. 10  $\mu$ L of the sample was run on a 15 7.5% analytical PAGE and bands were quantitated using a PhosphorImager. Results are calculated as a comparison to an "all ribo" control (non-modified nucleoside triphosphates) and the results are in Table IX.

Example 26: Incorporation of Multiple Modified Nucleoside triphosphates Into Oligonucleotides

Combinations of modified nucleoside triphosphates were tested with the 20 transcription protocol described in example 9, to determine the rates of incorporation of two or more of these triphosphates. Incorporation 2'-Deoxy-2'-(L-histidine) amino uridine (2'-his-NH<sub>2</sub>-UTP) was tested with unmodified cytidine nucleoside triphosphates, rATP and rGTP in reaction condition number 9. The data is presented as a percentage of incorporation of modified NTP's compared to the all rNTP control and is shown in Table 25 Xa.

Two modified cytidines (2'-NH<sub>2</sub>-CTP or 2'dCTP) were incorporated along with 2'-his-NH<sub>2</sub>-UTP with identical efficiencies. 2'-his-NH<sub>2</sub>-UTP and 2'-NH<sub>2</sub>-CTP were then tested with various unmodified and modified adenosine triphosphates in the same buffer

(Table Xb). The best modified adenosine triphosphate for incorporation with both 2'-his-NH<sub>2</sub>-UTP and 2'-NH<sub>2</sub>-CTP was 2'-NH<sub>2</sub>-DADTP.

EXAMPLE 27: Optimization of Reaction conditions for Incorporation of Modified Nucleotide Triphosphate

5 The combination of 2'-his-NH<sub>2</sub>-UTP, 2'-NH<sub>2</sub>-CTP, 2'-NH<sub>2</sub>-DAP, and rGTP was tested in several reaction conditions (Table XI) using the incorporation protocol described in example 14. The results demonstrate that of the buffer conditions tested, incorporation of these modified nucleoside triphosphates occur in the presence of both methanol and LiCl.

10 Example 28: Deprotection of Ribozyme in a 96 Well Plate

A ribozyme sequence (200nmole) was synthesized as described herein on a polystyrene solid support in a well of a 96 well plate. A 10:3:13 mixture (800  $\mu$ L) of anhydrous methylamine (308 $\mu$ L), triethylamine (92 $\mu$ L) and dimethylsulfoxide (DMSO) (400  $\mu$ L) was prepared of which half (400  $\mu$ L) was added to the well and incubated at room 15 temperature for 45 minutes. Following the reaction the solution was replaced with the remaining 400  $\mu$ L and incubated as before. At the end of the reaction, the solid support was filtered off, all 800  $\mu$ L of MA/TEA/DMSO solution was collected together and 100  $\mu$ L of TEA.3HF was added. The reaction was then heated at 65°C for 15 minutes and then cooled to room temperature. The solution was then quenched with aqueous 20 NH<sub>4</sub>HCO<sub>3</sub> (1mL) (see Figure 30). HPLC chromatography of the reaction mixture afforded 32 O. D.u<sub>260 nm</sub> of which 46% was full length ribozyme.

Example 29: Column Deprotection of Ribozyme

A ribozyme was synthesized using the column format as described herein. The polystyrene solid-support with protected oligoribonucleotide or modified 25 oligoribonucleotide (200 nmole) was transferred into a glass vial equipped with a screw cap. A 10:3:13 mixture of anhydrous methylamine ( 308  $\mu$ L), triethylamine (92  $\mu$ L) and dimethylsulfoxide (DMSO) (400  $\mu$ L) was added followed by vortexing of the glass vial. After allowing the reaction for 1.5 hours, the solid support was filtered off. 100  $\mu$ L of TEA.3HF was added at room temperature to the vial and the reaction was mixed causing

the solution to gel. The reaction was then heated at 65 °C for 15 minutes and then cooled to room temperature. The solution was then quenched with 1.5 M aqueous NH<sub>4</sub><sup>+</sup>HCO<sub>3</sub> (1mL). HPLC chromatography of the reaction mixture afforded 32 O. D.u<sub>260 nm</sub> of which 46% was full length ribozyme.

5 Example 30: Column Deprotection of Ribozyme with anhydrous ethanolic methylamine

A ribozyme was synthesized using the column format as described herein. The polystyrene solid-support with protected oligoribonucleotide or modified oligoribonucleotide (200 nmole) was transferred into a glass vial equipped with a screw cap. A 1:1 mixture of anhydrous ethanolic methylamine (400 µL) and dimethylsulfoxide (DMSO) (400 µL) was added followed by vortexing of the glass vial. After allowing the reaction for 1.5 hours, the solid support was filtered off. 100 µL of TEA.3HF was added at room temperature to the vial and the reaction was mixed causing the solution to gel. The reaction was then heated at 65 °C for 15 minutes and then cooled to room temperature. The solution was then quenched with 1.5 M aqueous NH<sub>4</sub><sup>+</sup>HCO<sub>3</sub> (1mL). HPLC chromatography of the reaction mixture afforded 32 O. D.u<sub>260 nm</sub> of which 46% was full length ribozyme.

Example 31. Large-scale One-Pot Deprotection of Ribozyme

A ribozyme was synthesized at the 0.5 mmol scale using the column format as described herein. The polystyrene solid-support (24 grs) with protected oligoribonucleotide or modified oligoribonucleotide (500 µmole) was transferred into a 1L Schott bottle equipped with a screw cap. A 1:1.3 mixture of anhydrous ethanolic methylamine (150 mL) and dimethylsulfoxide (DMSO) (200 mL) was added followed by vortexing (200 rpm) of the glass bottle for 1.5 hours. The reaction mixture was then frozen at -70 °C for 30 minutes. 50 mL of neat TEA.3HF was then added at room temperature to the reaction mixture and the reaction was placed in a shaking oven (200 rpm) where it was heated at 65 °C for 60 minutes and subsequently frozen at -70 °C for 30 minutes. The solution was then quenched with 1.5 M aqueous NH<sub>4</sub><sup>+</sup>HCO<sub>3</sub> (200 mL).

The reaction mixture was separated from the polystyrene solid-support by filtration on a sintered glass funnel (10-20  $\mu$ m porosity). U.V. spectrophotometric quantification and HPLC chromatography of the reaction mixture afforded 160,000 O.D.u<sub>260 nm</sub> of which 46.4% was full length ribozyme. After allowing the reaction for 1.5 hours, the solid support was filtered off

Example 32: Antitumor and antimetastatic efficacy of ribozymes directed against the mRNA encoding the two VEGF receptor subtypes, *flt-1* and *flk-1* in the mouse Lewis lung-HM carcinoma model of primary tumor growth and metastasis

The Lewis lung carcinoma (LLC) model is a syngeneic mouse model of metastatic cancer commonly used for antitumor agent efficacy screening. According to Folkman (1995, *supra*), primary tumor growth and metastasis in this model is dependent upon VEGF-induced angiogenesis. Two variants of the LLC model exist. The low metastatic form involves the implantation of a tumor, usually subcutaneous, which sends micrometastases to the lungs whose growth is suppressed by the presence of the primary tumor. The highly metastatic (HM) form differs from the low metastatic variant in that the growth of metastases is not suppressed by the presence of the primary tumor. Thus, the HM form is a model in which it is possible to measure pharmacologic efficacy on both primary tumor growth and metastasis in the same mouse without excision of the primary tumor.

Applicant selected the highly metastatic variant of the Lewis lung model for antitumor/metastatic screening of ribozymes directed against VEGF receptor (*flt-1* and *flk-1*) mRNA. These ribozymes have been shown to reduce VEGF binding and VEGF-stimulated proliferation in cultured MVEC's as well as VEGF-induced neovascularization of the rat cornea (Cushman et al., 1996, *Angiogenesis Inhibitors and Other Novel Therapeutic Strategies for Ocular Diseases of Neovascularization*, IBC Conference Abstract). Pharmacokinetically, Applicant has found that ribozymes distribute systemically following continuous i.v. infusion (*via* Alzet osmotic minipumps) at significant concentrations within most tissues including subcutaneously implanted

tumors. This study examines the antitumor/antimetastatic efficacy of *flt-1* and *flk-1* ribozymes continuously infused i.v. in the LLC-HM mouse model.

### Methods

#### *Ribozymes*

5 The ribozymes used in this study were hammerhead ribozymes comprising a 4 base pair stem II, four phosphorothioate linkages at the 5'-end, a 2'-C-allyl substitution at position 4 (see Figure 1), and an inverted abasic nucleotide substitution at the 3'-end. The catalytically active and inactive ribozymes were RPI.4610/4611 (active/inactive) and RPI.4733/4734 directed against *flt-1* and *flk-1* messages, respectively. Ribozymes 10 solutions were prepared in normal saline (USP).

Test solutions (ribozymes or saline control) were dispensed into Alzet® osmotic minipumps (Model # 1002--total volume capacity including excess = 200 µl) which dispense 0.5 µl/h at 37 °C when exposed to interstitial water. Pumps were either filled with normal saline (USP) or 167.0, 50.0, 16.7, 5.0, or 1.7 mg/ml ribozyme solutions. 15 Prior to animal implantation, osmotic minipumps were placed in 37 °C sterile water for at least four hours to activate pumping.

#### *Tumor inoculation*

All animal procedures in this study were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (1996), USDA 20 regulations, and the policies and procedures of the RPI Institutional Animal Care and Use Committee. A total of 210 female C57BL/6J mice weighing between 20-25 g were used in this study. All animals were housed under 12 h on/12 h off light cycles and received *ad libitum* food and water.

Highly metastatic variant Lewis lung carcinoma (LLC-HM) tumors were 25 propagated *in vivo* from an LLC-HM cell line. These tumors needed to be propagated *in vivo* because they can revert to the low metastatic phenotype in culture. LLC-HM cells

were initially cultured in DMEM + 10% FCS + 1 % GPS. For *in vivo* propagation, 5 X 10<sup>6</sup> cells were injected subcutaneously in mice. Tumors were allowed to grow for 25 days at which time animals were euthanized by CO<sub>2</sub> inhalation and lung macrometastases were counted. Animals with the most macrometastases (approximately 15-20) were 5 selected for preparation of tumor breis and propagation. When tumors in animals selected for propagation reached a volume of approximately 1500 mm<sup>3</sup>, animals were euthanized by CO<sub>2</sub> inhalation and tumors were excised. Tumors were sieved through a 100 µm pore size sterile nylon mesh. LLC-HM cells were resuspended in normal saline to a final concentration of 5 x 10<sup>6</sup> viable cells/ml (*via* hemocytometer). Three days prior to 10 ribozyme dosing, all animals were subcutaneously inoculated on the right flank with 5 x 10<sup>5</sup> cells (in a volume of 100 µl).

*Ribozyme or saline dosing*

Each ribozyme solution was prepared to deliver 100, 30, 10, 3, or 1 mg/kg/day in a volume of 12 ml. A total of 10 animals per dose or saline control group were surgically 15 implanted on the left flank with osmotic minipumps pre-filled with the respective test solution three days following tumor inoculation. Pumps were attached to indwelling jugular vein catheters. The specifications for the model #1002 Alzet osmotic minipump show that they accurately deliver aqueous solutions at 0.5 µl/h for 14 days. Table III summarizes the experimental groups.

20 *Tumor volume and metastatic index quantitation*

Beginning four days and ending 24 days days following tumor inoculation, the length and width of all primary tumors were measured every other day using microcalipers. Tumor volumes were calculated using the standard formula for an ellipsoid volume, (L·W<sup>2</sup>)/2. Tumor volumes were calculated in triplicate for each animal. A mean 25 tumor volume was calculated for each animal. Group means and standard error of the group means were calculated from individual animal mean tumor volumes.

Twenty-five days following tumor inoculation, all animals were euthanized by CO<sub>2</sub> inhalation and lungs and primary tumors harvested. Lung macrometastases were counted under a dissecting microscope (2.5 X magnification). Lungs and primary tumors were also weighed on an analytical balance. Lung weights served as an index of total lung 5 metastatic burden.

#### *Statistical analysis*

For all treatment groups, group tumor volume means on day 18 (end of treatment) as well as means of primary tumor and lung weight and numbers of lung metastases were evaluated for normality and subjected to analysis of variance. Statistical differences 10 between group means were evaluated using the Tukey-Kramer post-hoc test (alpha = 0.05). Comparisons with the control group (saline control) were made using the Dunnett's test (alpha = 0.05).

#### Results

##### *Flt-1*

15 The effects of several doses of active and inactive *Flt-1* ribozymes (RPI.4610/4611, respectively) on primary LLC-HM tumor growth are summarized in Figure 39 (A-E). At the lowest dose (Figure 39A), both active and inactive reduce primary tumor growth similarly throughout the entire time course compared to saline controls. However, with increasing dose, active ribozyme reduces primary tumor growth to a greater extent than 20 the inactive ribozyme, with the largest difference observed at 30 mg/kg/day (Figure 39D). The magnitude of the maximal reduction compared to saline was approximately four fold with the active ribozyme RPI.4610 at 30 mg/kg/day. It should be noted that this observed four fold reduction is still present at day 24 even though treatment ended 7 days earlier.

25 The growth curve data was subjected to exponential regression. The curve fits show that the tumor growth data fits an exponential curve with a high correlation coefficient (R>0.95). Thus, there appears to be a long lasting toxic effect on tumor growth. Since

the calculated slope of the exponential curve at any point indicates the rate of tumor growth, it should be possible to compare rates of growth between treatments. Since the curve fits do not assume that the tumor growth starts from the same point (which is a correct assumption since the all tumors start with the same tumor cell inoculum 5 concentration), an accurate calculation of the slope of the exponential curve is not possible since the curve fitting algorithm extrapolates a  $t = 0$  tumor size which is then used to calculate the slope. In the analysis, the saline tumor size at  $t = 0$  is much greater than the other treatment groups, thus comparisons with saline are not necessarily accurate. If the curve fit algorithm is restricted to the same tumor size, a dose-dependent reduction 10 in the rate of tumor growth is observed with the active ribozyme. However, the curve fits show lower correlation coefficients in some cases.

In order to see whether a the ribozyme treatments statistically reduce primary tumor growth, primary tumor volume measurements at each dose immediately following treatment (day 18) were compared (Figure 40). Active ribozyme RPI.4610 produced a 15 statistically significant ( $p < 0.05$ ) and dose-dependent reduction in primary tumor volume. Although the inactive ribozyme (RPI.4611) showed some reduction in primary tumor volume at the lowest and highest doses, there was no dose-dependent reduction observed. At doses between 3 and 30 mg/kg/day, the inactive ribozyme showed no significant 20 reduction in primary tumor volume. There was a significant difference ( $p < 0.05$ ) between active and inactive ribozymes (Tukey-Kramer test) at doses of 10 and 30 mg/kg/day.

Applicant has also observed that the active ribozyme RPI.4610 produced a significant reduction in primary tumor mass at all doses tested (1-100 mg/kg/day) 25 days following inoculation.

25 Figures 41 A and B illustrate that the active ribozyme reduced both the number of lung metastases and lung mass in a dose-dependent manner. The active *flt-1* ribozyme showed a significant reduction ( $p < 0.05$  by Dunnett's) in the number of lung metastases at the 30 and 100 mg/kg/day doses compared to saline. There was also a significant

difference between active and inactive ribozymes at these doses ( $p > 0.05$  by Student's t). RPI.4610 reduced the lung weight to almost normal levels at the highest dose (100 mg/kg/day). There was no observable dose-related effect of the inactive ribozyme on either the number of lung metastases or lung weight. A significant reduction ( $p < 0.05$ , 5 Student's t) in lung mass, an index of metastatic burden, was observed between saline and the active ribozyme. The lack of significance using more stringent statistical tests (Dunnet's or Tukey-Kramer), which take into account the variance within all groups, was due to high variability, especially in the inactive ribozyme group. However, since five doses were tested, it is possible to say that there is a dose-dependent trend in the reduction 10 of lung metastases/lung weight.

Example 33: Effects of *flk-1* ribozymes (active/inactive) on LLC-HM primary tumor growth in mice.

The dose-related effects of active and inactive *flk-1* directed ribozymes (RPI.4733/4734, respectively) on primary LLCare shown in Figure 38 A-E.

15 The dose-related effects of active and inactive *flk-1* directed ribozymes (RPI.4733/4734, respectively) on primary LLCare shown in Figure 42 A-E. At the lowest dose, there was no observable effect on primary tumor growth with the active *flk-1* ribozyme (Figure 42A). The inactive ribozyme showed a modest reduction in primary tumor growth. At higher doses (3-100 mg/kg/day, Figure 42B-E), the active *flk-1* 20 ribozyme reduced primary tumor growth while the inactive ribozyme showed little, if any, antitumor efficacy over the dose range between 10 and 100 mg/kg/day (Figures 42C-E). The antitumor efficacy of both active and inactive *flk-1* ribozymes are similar at 3 mg/kg/day (Figure 42B).

25 As in the case of the *flt-1* ribozymes, tumor growth followed exponential growth kinetics. Again, since the  $t = 0$  tumor size could not accurately be estimated by the curve fit program, it is not possible to calculate the slope of the exponential curve fits for the *flk-1* ribozymes.

Immediately following the cessation of treatment (day 18), the active *flk-1* ribozyme showed a significant reduction in primary tumor volume from 3 to 100 mg/kg/day (Figure 43). The magnitude of the reduction is approximately four fold and appeared to be maximal at 3 mg/kg/day. The lowest dose had no significant effect on primary tumor 5 volume. The inactive *flk-1* ribozyme had a significant antitumor effect at doses of 1 and 3 mg/kg/day; however, this effect disappeared between 10 and 100 mg/kg/day.

The antimetastatic effects of the *flk-1* ribozymes are illustrated in Figure 44 A and B. Although neither ribozyme showed a statistically significant effect on the number of lung metastases at any dose, it appears that the active *flk-1* ribozyme showed a significant 10 reduction in lung mass over the dose range between 3 and 100 mg/kg/day.

Applicant has further observed that the lung mass was reduced to normal over the entire dose range. The inactive ribozyme reduced lung mass at 1 and 3 mg/kg/day (Figure 41C); however, this trend was not observed at higher doses (3-100 mg/kg/day).

Example 34: Ribozyme-mediated decrease in vascularity of tumor

15 Three tumors from each of three treatment groups (saline controls, inactive RPI.4611 and active RPI.4610, 30 mg/kg/day dose only) were analyzed for vascularity using an immunohistochemical assay which stains endothelial cells for CD31 (PECAM). The vascularity was quantitated in a blinded fashion. From the raw data the average 20 number of vessels per high magnification field (400X) were calculated. They are as follows: SALINE CONTROL = 24.1; RPI.4611 (Inactive) = 27.6; RPI.4610 (Active) = 16.0.

It is suggestive that ribozyme-specific antiangiogenic effect is exhibited by the active Flt-1 ribozyme in Lewis lung tumors. Thus, the mechanism of action for the observed reduction in the primary tumor volumes may be due to an antiangiogenic effect. 25 Similar delivery strategies can be used to deliver c-raf ribozymes to treat a variety of diseases.

Use of Ribozymes Targeting *c-raf*

Overexpression of the *c-raf* oncogene has been reported in a number of cancers (see above). Thus, inhibition of *c-raf* expression (for example using ribozymes) can reduce cell proliferation of a number of cancers, *in vitro* and *in vivo* and can reduce their 5 proliferative potential. A cascade of MMP and serine proteinase expression is implicated in the acquisition of an invasive phenotype as well as in angiogenesis in tumors (MacDougall & Matrisian, 1995, *Cancer & Metastasis Reviews* 14, 351;Ritchlin & Winchester, 1989, *Springer Semin Immunopathol.*, 11, 219).

A number of human diseases are characterized by the inappropriate proliferation 10 of cells at sites of injury or damage to the normal tissue architecture. These diseases include restenosis, caused by the local proliferation of medial smooth muscle cells at sites of arterial wall disruption by surgery; psoriasis, caused by proliferation of keratinocytes at regions of endothelial cell damage in the skin, and various fibrosis, caused by the inappropriate replication of cells during wound healing processes. In certain 15 inflammatory processes, cell proliferation may not be causative, yet it exacerbates the disease pathology. For example, in rheumatoid arthritis, synovial hyperplasia leads to accelerated cartilage damage due to secretion of proteases by the expanding population of synovial fibroblasts. Any number of these diseases and others which involve cellular proliferation or the loss of proliferative control, such as cancer, could be treated using 20 ribozymes which inhibit the expression of the cellular *Raf* gene products. Alternatively, ribozyme inhibition of the cellular growth factor receptors could be used to inhibit downstream signalling pathways. The specific growth factors involved would depend upon the cell type indicated in the proliferative event.

Ribozymes, with their catalytic activity and increased site specificity (see above), 25 are likely to represent a potent and safe therapeutic molecule for the treatment of cancer. In the present invention, ribozymes are shown to inhibit smooth muscle cell proliferation. From those practiced in the art, it is clear from the examples described, that the same

ribozymes may be delivered in a similar fashion to cancer cells to block their proliferation.

Diagnostic uses

Ribozymes of this invention may be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of *c-raf* RNA in a cell. The close relationship between ribozyme activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple ribozymes described in this invention, one may map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with ribozymes may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combinational therapies (e.g., multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules). Other *in vitro* uses of ribozymes of this invention are well known in the art, and include detection of the presence of mRNAs associated with *c-raf* related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a ribozyme using standard methodology.

In a specific example, ribozymes which can cleave only wild-type or mutant forms of the target RNA are used for the assay. The first ribozyme is used to identify wild-type RNA present in the sample and the second ribozyme will be used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA will be cleaved by both ribozymes to demonstrate the relative ribozyme efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates will also serve to generate size markers

for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis will require two ribozymes, two substrates and one unknown sample which will be combined into six reactions. The presence of cleavage products will be determined using an RNase protection assay so that full-length and cleavage fragments of each RNA 5 can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, *c-ras*) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, 10 then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios will be correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

#### Additional Uses

Potential usefulness of sequence-specific nucleic acid catalysts of the instant 15 invention might have many of the same applications for the study of RNA that DNA restriction endonucleases have for the study of DNA (Nathans *et al.*, 1975 *Ann. Rev. Biochem.* 44:273). For example, the pattern of restriction fragments could be used to establish sequence relationships between two related RNAs, and large RNAs could be specifically cleaved to fragments of a size more useful for study. The ability to engineer 20 sequence specificity of the ribozyme is ideal for cleavage of RNAs of unknown sequence.

The use of NTP's described in this invention have several research and commercial applications. These modified nucleoside triphosphates can be used for *in vitro* selection (evolution) of oligonucleotides with novel functions. Examples of *in vitro* selection protocols are incorporated herein by reference (Joyce, 1989, *Gene*, 82, 83-87; 25 Beaudry *et al.*, 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.*, 1994, *TIBTECH* 12, 268; Bartel *et al.*, 1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.*, 1995, *FASEB J.*, 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442).

Additionally, these modified nucleoside triphosphates can be employed to generate modified oligonucleotide combinatorial chemistry libraries. Several references for this technology exist (Brenner *et al.*, 1992, *PNAS* 89, 5381-5383, Eaton, 1997, *Curr. Opin. Chem. Biol.* 1, 10-16) which are all incorporated herein by reference.

5 Nucleic acid molecules of the instant invention might have many of the same applications for the study of RNA that DNA restriction endonucleases have for the study of DNA (Nathans *et al.*, 1975 *Ann. Rev. Biochem.* 44:273). For example, the pattern of restriction fragments could be used to establish sequence relationships between two related RNAs, and large RNAs could be specifically cleaved to fragments of a size more 10 useful for study. The ability to engineer sequence specificity of the ribozyme is ideal for cleavage of RNAs of unknown sequence. Nucleic acid molecules (e.g., ribozymes) of the invention can be used, for example, to target cleavage of virtually any RNA transcript (Zaug *et al.*, 324, *Nature* 429 1986 ; Cech, 260 *JAMA* 3030, 1988; and Jefferies *et al.*, 17 *Nucleic Acids Research* 1371, 1989). Such nucleic acids can be used as a therapeutic or 15 to validate a therapeutic gene target and/or to determine the function of a gene in a biological system (Christoffersen, 1997, *Nature Biotech.* 15, 483).

Various ligands can be attached to oligonucleotides using the compounds containing 20 zylo modification for the purposes of cellular delivery, nuclease resistance, cellular trafficking and localization, chemical ligation of oligonucleotide fragments. Incorporation of one or more compounds of Formula II into a ribozyme may increase its effectiveness. Compounds of Formula II can be used as potential antiviral agents.

Other embodiments are within the following claims.

## TABLE I

## Characteristics of naturally occurring ribozymes

## Group I Introns

5     •     Size: ~150 to >1000 nucleotides.

•     Requires a U in the target sequence immediately 5' of the cleavage site.

•     Binds 4-6 nucleotides at the 5'-side of the cleavage site.

•     Reaction mechanism: attack by the 3'-OH of guanosine to generate cleavage products with 3'-OH and 5'-guanosine.

10    •     Additional protein cofactors required in some cases to help folding and maintainance of the active structure.

•     Over 300 known members of this class. Found as an intervening sequence in *Tetrahymena thermophila* rRNA, fungal mitochondria, chloroplasts, phage T4, blue-green algae, and others.

15    •     Major structural features largely established through phylogenetic comparisons, mutagenesis, and biochemical studies [<sup>1,2</sup>].

•     Complete kinetic framework established for one ribozyme [<sup>3,4,5,6</sup>].

•     Studies of ribozyme folding and substrate docking underway [<sup>7,8,9</sup>].

•     Chemical modification investigation of important residues well established [<sup>10,11</sup>].

<sup>1</sup> Michel, Francois; Westhof, Eric. Slippery substrates. *Nat. Struct. Biol.* (1994), 1(1), 5-7.

<sup>2</sup> Lisacek, Frederique; Diaz, Yolande; Michel, Francois. Automatic identification of group I intron cores in genomic DNA sequences. *J. Mol. Biol.* (1994), 235(4), 1206-17.

<sup>3</sup> Herschlag, Daniel; Cech, Thomas R.. Catalysis of RNA cleavage by the *Tetrahymena thermophila* ribozyme. 1. Kinetic description of the reaction of an RNA substrate complementary to the active site. *Biochemistry* (1990), 29(44), 10159-71.

<sup>4</sup> Herschlag, Daniel; Cech, Thomas R.. Catalysis of RNA cleavage by the *Tetrahymena thermophila* ribozyme. 2. Kinetic description of the reaction of an RNA substrate that forms a mismatch at the active site. *Biochemistry* (1990), 29(44), 10172-80.

<sup>5</sup> Knitt, Deborah S.; Herschlag, Daniel. pH Dependencies of the *Tetrahymena* Ribozyme Reveal an Unconventional Origin of an Apparent pKa. *Biochemistry* (1996), 35(5), 1560-70.

<sup>6</sup> Bevilacqua, Philip C.; Sugimoto, Naoki; Turner, Douglas H.. A mechanistic framework for the second step of splicing catalyzed by the *Tetrahymena* ribozyme. *Biochemistry* (1996), 35(2), 648-58.

<sup>7</sup> Li, Yi; Bevilacqua, Philip C.; Mathews, David; Turner, Douglas H.. Thermodynamic and activation parameters for binding of a pyrene-labeled substrate by the *Tetrahymena* ribozyme: docking is not diffusion-controlled and is driven by a favorable entropy change. *Biochemistry* (1995), 34(44), 14394-9.

<sup>8</sup> Banerjee, Aloke Raj; Turner, Douglas H.. The time dependence of chemical modification reveals slow steps in the folding of a group I ribozyme. *Biochemistry* (1995), 34(19), 6504-12.

<sup>9</sup> Zarrinkar, Patrick P.; Williamson, James R.. The P9.1-P9.2 periph ral extension h lps guide folding of the *Tetrahymena* ribozyme. *Nucleic Acids Res.* (1996), 24(5), 854-8.

<sup>10</sup> Strobel, Scott A.; Cech, Thomas R.. Minor groove recognition f the cons rved G.cntdot.U pair at the *Tetrahymena* ribozym reaction site. *Science* (Washington, D. C.) (1995), 267(5198),

- The small (4-6 nt) binding site may make this ribozyme too non-specific for targeted RNA cleavage, however, the Tetrahymena group I intron has been used to repair a "defective"  $\beta$ -galactosidase message by the ligation of new  $\beta$ -galactosidase sequences onto the defective message [<sup>12</sup>].

5

### RNAse P RNA (M1 RNA)

- Size: ~290 to 400 nucleotides.
- RNA portion of a ubiquitous ribonucleoprotein enzyme.
- 10 • Cleaves tRNA precursors to form mature tRNA [<sup>13</sup>].
- Reaction mechanism: possible attack by  $M^{2+}$ -OH to generate cleavage products with 3'-OH and 5'-phosphate.
- RNAse P is found throughout the prokaryotes and eukaryotes. The RNA subunit has been sequenced from bacteria, yeast, rodents, and primates.
- 15 • Recruitment of endogenous RNAse P for therapeutic applications is possible through hybridization of an External Guide Sequence (EGS) to the target RNA [<sup>14,15</sup>]
- Important phosphate and 2' OH contacts recently identified [<sup>16,17</sup>]

### Group II Introns

- 20 • Size: >1000 nucleotides.
- Trans cleavage of target RNAs recently demonstrated [<sup>18,19</sup>].
- Sequence requirements not fully determined.
- Reaction mechanism: 2'-OH of an internal adenosine generates cleavage products with 3'-OH and a "lariat" RNA containing a 3'-5' and a 2'-5' branch point.

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675-9.

<sup>11</sup> Strobel, Scott A.; Cech, Thomas R.. Exocyclic Amine of the Conserved G.cndot.U Pair at the Cleavage Site of the Tetrahymena Ribozyme Contributes to 5'-Splice Site Selection and Transition State Stabilization. *Biochemistry* (1996), 35(4), 1201-11.

<sup>12</sup> Sullenger, Bruce A.; Cech, Thomas R.. Ribozyme-mediated repair of defective mRNA by targeted trans-splicing. *Nature (London)* (1994), 371(6498), 619-22.

<sup>13</sup> Robertson, H.D.; Altman, S.; Smith, J.D. *J. Biol. Chem.*, 247, 5243-5251 (1972).

<sup>14</sup> Forster, Anthony C.; Altman, Sidney. External guide sequences for an RNA enzyme. *Science (Washington, D. C.)*, 1883- (1990), 249(4970), 783-6.

<sup>15</sup> Yuan, Y.; Hwang, E. S.; Altman, S. Targeted cleavage of mRNA by human RNase P. *Proc. Natl. Acad. Sci. USA* (1992) 89, 8006-10.

<sup>16</sup> Harris, Michael E.; Pace, Norman R.. Identification of phosphates involved in catalysis by the ribozyme RNase P RNA. *RNA* (1995), 1(2), 210-18.

<sup>17</sup> Pan, Tao; Loria, Andrew; Zhong, Kun. Probing of tertiary interactions in RNA: 2'-hydroxyl-base contacts between the RNase P RNA and pre-tRNA. *Proc. Natl. Acad. Sci. U. S. A.* (1995), 92(26), 12510-14.

<sup>18</sup> Pyle, Anna Marie; Green, Justin B.. Building a Kinetic Framework for Group II Intron Ribozyme Activity: Quantitation of Interdomain Binding and Reaction Rate. *Biochemistry* (1994), 33(9), 2716-25.

<sup>19</sup> Micheis, William J. Jr.; Pyle, Anna Marie. Conversion of a Group II Intron into a New Multiple-Turnover Ribozyme that Selectively Cleaves Oligonucleotides: Elucidation of Reaction Mechanism and Structure/Function Relationships. *Biochemistry* (1995), 34(9), 2965-77.

- Only natural ribozyme with demonstrated participation in DNA cleavage [<sup>20,21</sup>] in addition to RNA cleavage and ligation.
- Major structural features largely established through phylogenetic comparisons [<sup>22</sup>].
- Important 2' OH contacts beginning to be identified [<sup>23</sup>]
- 5 • Kinetic framework under development [<sup>24</sup>]

### Neurospora VS RNA

- Size: ~144 nucleotides.
- 10 • Trans cleavage of hairpin target RNAs recently demonstrated [<sup>25</sup>].
- Sequence requirements not fully determined.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Binding sites and structural requirements not fully determined.
- 15 • Only 1 known member of this class. Found in Neurospora VS RNA.

### Hammerhead Ribozyme

(see text for references)

- Size: ~13 to 40 nucleotides.
- 20 • Requires the target sequence UH immediately 5' of the cleavage site.
- Binds a variable number nucleotides on both sides of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 14 known members of this class. Found in a number of plant pathogens (virusoids) that use RNA as the infectious agent.
- 25 • Essential structural features largely defined, including 2 crystal structures [<sup>26,27</sup>]
- Minimal ligation activity demonstrated (for engineering through *in vitro* selection) [<sup>28</sup>]

<sup>20</sup> Zimmerly, Steven; Guo, Huatao; Eskes, Robert; Yang, Jian; Perlman, Philip S.; Lambowitz, Alan M.. A group II intron RNA is a catalytic component of a DNA endonuclease involved in intron mobility. *Cell* (Cambridge, Mass.) (1995), 83(4), 529-38.

<sup>21</sup> Griffin, Edmund A., Jr.; Qin, Zifeng; Michels, Williams J., Jr.; Pyle, Anna Marie. Group II intron ribozymes that cleave DNA and RNA linkages with similar efficiency, and lack contacts with substrate 2'-hydroxyl groups. *Chem. Biol.* (1995), 2(11), 761-70.

<sup>22</sup> Michel, Francois; Ferat, Jean Luc. Structure and activities of group II introns. *Annu. Rev. Biochem.* (1995), 64, 435-61.

<sup>23</sup> Abramovitz, Dana L.; Friedman, Richard A.; Pyle, Anna Marie. Catalytic role of 2'-hydroxyl groups within a group II intron active site. *Science* (Washington, D. C.) (1996), 271(5254), 1410-13.

<sup>24</sup> Daniels, Danette L.; Michels, William J., Jr.; Pyle, Anna Marie. Two competing pathways for self-splicing by group II introns: a quantitative analysis of *in vitro* reaction rates and products. *J. Mol. Biol.* (1996), 256(1), 31-49.

<sup>25</sup> Guo, Hans C. T.; Collins, Richard A.. Efficient trans-cleavage of a stem-loop RNA substrate by a ribozyme derived from *Neurospora* VS RNA. *EMBO J.* (1995), 14(2), 368-76.

<sup>26</sup> Scott, W.G., Finch, J.T., Aaron, K. The crystal structure of an all RNA hammerhead ribozyme: A proposed mechanism for RNA catalytic cleavage. *Cell*, (1995), 81, 991-1002.

<sup>27</sup> McKay, Structure and function of the hammerhead ribozyme: an unfinished story. *RNA*, (1996), 2, 395-403.

<sup>28</sup> Long, D., Uhlenbeck, O., Hertel, K. Ligation with hammerhead ribozymes. US Patent No.

- Complete kinetic framework established for two or more ribozymes [<sup>29</sup>].
- Chemical modification investigation of important residues well established [<sup>30</sup>].

### Hairpin Ribozyme

5

- Size: ~50 nucleotides.
- Requires the target sequence GUC immediately 3' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site and a variable number to the 3'-side of the cleavage site.

10

- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 3 known members of this class. Found in three plant pathogen (satellite RNAs of the tobacco ringspot virus, arabis mosaic virus and chicory yellow mottle virus) which uses RNA as the infectious agent.

15

- Essential structural features largely defined [<sup>31, 32, 33, 34</sup>]
- Ligation activity (in addition to cleavage activity) makes ribozyme amenable to engineering through *in vitro* selection [<sup>35</sup>]
- Complete kinetic framework established for one ribozyme [<sup>36</sup>].
- Chemical modification investigation of important residues begun [<sup>37, 38</sup>].

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5,633,133.

<sup>29</sup> . Hertel, K.J., Herschlag, D., Uhlenbeck, O. A kinetic and thermodynamic framework for the hammerhead ribozyme reaction. *Biochemistry*, (1994) 33, 3374-3385. Beigelman, L., et al., Chemical modifications of hammerhead ribozymes. *J. Biol. Chem.*, (1995) 270, 25702-25708.

<sup>30</sup> . Beigelman, L., et al., Chemical modifications of hammerhead ribozymes. *J. Biol. Chem.*, (1995) 270, 25702-25708.

<sup>31</sup> . Hampel, Arnold; Tritz, Richard; Hicks, Margaret; Cruz, Phillip. 'Hairpin' catalytic RNA model: evidence for helices and sequence requirement for substrate RNA. *Nucleic Acids Res.* (1990), 18(2), 299-304.

<sup>32</sup> . Chowrira, Bharat M.; Berzal-Herranz, Alfredo; Burke, John M.. Novel guanosine requirement for catalysis by the hairpin ribozyme. *Nature (London)* (1991), 354(6351), 320-2.

<sup>33</sup> . Berzal-Herranz, Alfredo; Joseph, Simpson; Chowrira, Bharat M.; Butcher, Samuel E.; Burke, John M.. Essential nucleotide sequences and secondary structure elements of the hairpin ribozyme. *EMBO J.* (1993), 12(6), 2567-73.

<sup>34</sup> . Joseph, Simpson; Berzal-Herranz, Alfredo; Chowrira, Bharat M.; Butcher, Samuel E.. Substrate selection rules for the hairpin ribozyme determined by *in vitro* selection, mutation, and analysis of mismatched substrates. *Genes Dev.* (1993), 7(1), 130-8.

<sup>35</sup> . Berzal-Herranz, Alfredo; Joseph, Simpson; Burke, John M.. *In vitro* selection of active hairpin ribozymes by sequential RNA-catalyzed cleavage and ligation reactions. *Genes Dev.* (1992), 6(1), 129-34.

<sup>36</sup> . Hegg, Lisa A.; Fedor, Martha J.. Kinetics and Thermodynamics of Intermolecular Catalysis by Hairpin Ribozymes. *Biochemistry* (1995), 34(48), 15813-28.

<sup>37</sup> . Grasby, Jane A.; Mersmann, Karin; Singh, Mohinder; Gait, Michael J.. Purine Functional Groups in Essential Residues of the Hairpin Ribozyme Required for Catalytic Cleavage of RNA. *Biochemistry* (1995), 34(12), 4068-76.

<sup>38</sup> . Schmidt, Sabine; Beigelman, Leonid; Karpeisky, Alexander; Usman, Nassim; Sorensen, Ulrik S.; Gait, Michael J.. Base and sugar requirements for RNA cleavage of essential nucleosid residues in internal loop B of the hairpin ribozyme: implications for secondary structure. *Nucleic Acids Res.* (1996), 24(4), 573-81.

**Hepatitis Delta Virus (HDV) Ribozyme**

- Size: ~60 nucleotides.
- Trans cleavage of target RNAs demonstrated [39].
- 5 • Binding sites and structural requirements not fully determined, although no sequences 5' of cleavage site are required. Folded ribozyme contains a pseudoknot structure [40].
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Only 2 known members of this class. Found in human HDV.
- 10 • Circular form of HDV is active and shows increased nuclease stability [41]

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<sup>39</sup> Perrotta, Anne T.; Been, Michael D.. Cleavage of oligoribonucleotides by a ribozyme derived from the hepatitis delta virus RNA sequence. *Biochemistry* (1992), 31(1), 16-21.

<sup>40</sup> Perrotta, Anne T.; Been, Michael D.. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. *Nature (London)* (1991), 350(6317), 434-6.

<sup>41</sup> Puttaraju, M.; Perrotta, Anne T.; Been, Michael D.. A circular trans-acting hepatitis delta virus ribozyme. *Nucleic Acids Res.* (1993), 21(18), 4253-8.

Table II: 2.5  $\mu$ mol RNA Synthesis Cycle

Reagent	Equivalents	Amount	Wait Time*
Phosphoramidites	6.5	163 $\mu$ L	2.5
<i>S</i> -Ethyl Tetrazole	23.8	238 $\mu$ L	2.5
Acetic Anhydride	100	233 $\mu$ L	5 sec
<i>N</i> -Methyl Imidazole	186	233 $\mu$ L	5 sec
TCA	83.2	1.73 mL	21 sec
Iodine	8.0	1.18 mL	45 sec
Acetonitrile	NA	6.67 mL	NA

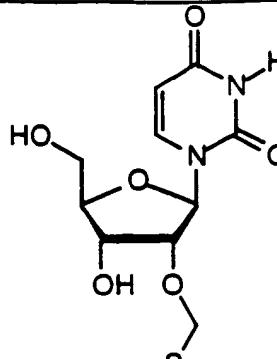
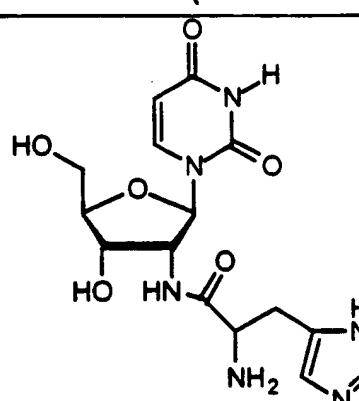
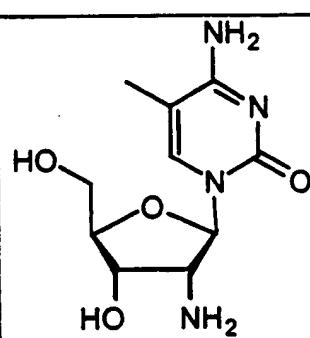
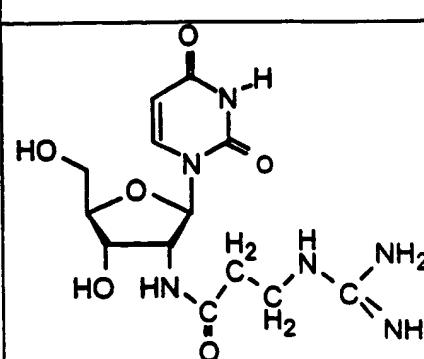
\* Wait time does not include contact time during delivery.

**TABLE III. NUCLEOSIDES USED FOR CHEMICAL SYNTHESIS  
OF MODIFIED NUCLEOTIDE TRIPHOSPHATES**

	NUCLEOSIDES	Abbreviation	CHEMICAL STRUCTURE
1	2'- <i>O</i> -methyl-2,6-diaminopurine riboside	2'- <i>O</i> -Me-DAP	
2	2'-deoxy-2'amino-2,6-diaminopurine riboside	2'-NH <sub>2</sub> -DAP	
3	2'-( <i>N</i> -alanyl)amino-2'-deoxy-uridine	ala-2'-NH <sub>2</sub> -U	
4	2'-( <i>N</i> -phenylalanyl)amino-2'-deoxy-uridine	phe-2'-NH <sub>2</sub> -U	

	NUCLEOSIDES	Abbreviation	CHEMICAL STRUCTURE
5	2'-( <i>N</i> -β-alanyl) amino-2'-deoxy uridine	2'-β-Ala-NH <sub>2</sub> -U	
6	2'-Deoxy-2'-(lysyl) amino uridine	2'-L-lys-NH <sub>2</sub> -U	
7	2'- <i>C</i> -allyl uridine	2'-C-allyl-U	
8	2'- <i>O</i> -amino-uridine	2'-O-NH <sub>2</sub> -U	

	NUCLEOSIDES	Abbreviation	CHEMICAL STRUCTURE
9	2'-O-methylthiomethyl adenosine	2'-O-MTM-A	
10	2'-O-methylthiomethyl cytidine	2'-O-MTM-C	
11	2'-O-methylthiomethyl guanosine	2'-O-MTM-G	

	NUCLEOSIDES	Abbreviation	CHEMICAL STRUCTURE
12	2'-O-methylthiomethyl-uridine	2'-O-MTM-U	
13	2'-(N-histidyl) amino uridine	2'-his-NH <sub>2</sub> -U	
14	2'-Deoxy-2'-amino-5-methyl cytidine	5-Me-2'-NH <sub>2</sub> -C	
15	2'-(N-β-carboxamidine-β-alanyl)amino-2'-deoxy-uridine	β-ala-CA-NH <sub>2</sub> -U	

	NUCLEOSIDES	Abbreviation	CHEMICAL STRUCTURE
16	2'-( <i>N</i> -β-alanyl) guanosine	β-Ala-NH <sub>2</sub> -G	
17	2'- <i>O</i> -Amino-Adenosine	2'- <i>O</i> -NH <sub>2</sub> -A	

Table VI. PHOSPHORYLATION OF URIDINE IN THE PRESENCE OF DMAP

0 equiv. DMAP		0.2 equiv. DMAP		0.5 equiv. DMAP		1.0 equiv. DMAP	
Time (min)	Product %	Time (min)	Product %	Time (min)	Product %	Time (min)	Product %
0	1	0	0	0	0	0	0
40	7	10	8	20	27	30	74
80	10	50	24	60	46	70	77
120	12	90	33	100	57	110	84
160	14	130	39	140	63	150	83
200	17	170	43	180	63	190	84
240	19	210	47	220	64	230	77
320	20	250	48	260	68	270	79
<b>1130</b>	<b>48</b>	290	49	300	64	310	77
1200	46	1140	68	1150	76	1160	72
		1210	69	1220	76	1230	74

C condition No.	TRIS-HCL (mM)	MgCl <sub>2</sub> (mM)	DTT (mM)	Spermidine (mM)	Triton X-100 (%)	METHANOL (%)	LiCl (mM)	PEG (%)	Temp(°C)
1	40 (pH 8.0)	20	10	5	0.01	10	1	-	25
2	40 (pH 8.0)	20	10	5	0.01	10	1	4	25
3	40 (pH 8.1)	12	5	1	0.002	-	-	4	25
4	40 (pH 8.1)	12	5	1	0.002	10	-	4	25
5	40 (pH 8.1)	12	5	1	0.002	-	1	4	25
6	40 (pH 8.1)	12	5	1	0.002	10	1	4	25
7	40 (pH 8.0)	20	10	5	0.01	10	1	-	37
8	40 (pH 8.0)	20	10	5	0.01	10	1	4	37
9	40 (pH 8.1)	12	5	1	0.002	-	-	4	37
10	40 (pH 8.1)	12	5	1	0.002	10	-	4	37
11	40 (pH 8.1)	12	5	1	0.002	-	1	4	37
12	40 (pH 8.1)	12	5	1	0.002	10	1	4	37

Table VII. Detailed Description of the NTP Incorporation Reaction Conditions

Modification	COND# 1	COND# 2	COND# 3	COND# 4	COND# 5	COND# 6	COND# 7	COND# 8	COND# 9	COND# 10	COND# 11	COND# 12
2'-NH <sub>2</sub> -ATP	1	2	3	5	2	4	1	2	10	11	5	9
2'-NH <sub>2</sub> -CTP	11	37	45	64	25	70	26	54	292	264	109	244
2'-NH <sub>2</sub> -GTP	4	7	6	14	5	17	3	16	10	21	9	16
2'-NH <sub>2</sub> -UTP	14	45	4	100	85	82	48	88	20	418	429	440
2'-dATP	9	3	19	23	9	24	6	3	84	70	28	51
2'-dCTP	1	10	43	46	35	47	27	127	204	212	230	235
2'-dGTP	6	10	9	15	9	12	8	34	38	122	31	46
2'-dTTP	9	9	14	18	13	18	8	15	116	114	59	130
2'-O-Me-ATP	0	0	0	0	0	0	1	1	2	2	2	2
2'-O-Me-CTP	no data compared to ribo; incorporates at low level											
2'-O-M-GTP	4	3	4	4	4	4	2	4	4	4	5	5
2'-O-M-UTP	55	52	39	38	41	48	55	71	93	103	81	77
2'-O-M-JUTP	4	4	3	4	4	5	4	3	4	5	5	5
2'-O-M-DAP	0	0	1	1	1	1	1	0	0	0	0	0
2'-NH <sub>2</sub> -DAP	2	2	2	2	3	4	14	18	15	20	13	14
ala-2'-NH <sub>2</sub> -UTP	8	12	7	7	8	8	4	10	6	6	10	6
phe-2'-NH <sub>2</sub> -UTP	65	48	25	17	21	220	223	265	300	275	248	
2'-B-NH <sub>2</sub> -ala-UTP	227	252	98	103	100	116	288	278	471	198	317	185
2'-F-ATP	39	44	17	30	17	26	172	130	375	447	377	438
2'-F-GTP	3	2	2	3	3	2	3	3	2	3	3	3
2'-Callyl-UTP	6	8	5	5	4	5	16	23	24	24	19	24
2'-O-NH <sub>2</sub> -UTP	0	1	0	0	0	0	1	0	0	0	0	0
2'-O-MTM-ATP	2	2	1	1	1	1	3	4	5	4	5	3
2'-O-MTM-CTP	6	1	1	3	1	2	0	1	1	3	1	4
2'-O-MTM-GTP												

Table VIII. INCORPORATION OF MODIFIED NUCLEOTIDE TRIPHOSPHATES

**Table IX: INCORPORATION OF MODIFIED  
NUCLEOTIDE TRIPHOSPHATES USING WILD TYPE  
BACTERIOPHAGE T7 POLYMERASE**

Modification	label	% ribo control
2'-NH <sub>2</sub> -GTP	ATP	4%
2'-dGTP	ATP	3%
2'-O-Me-GTP	ATP	3%
2'-F-GTP	ATP	4%
2'-O-MTM-GTP	ATP	3%
2'-NH <sub>2</sub> -UTP	ATP	39%
2'-dTTP	ATP	5%
2'-O-Me-UTP	ATP	3%
ala-2'-NH <sub>2</sub> -UTP	ATP	2%
phe-2'-NH <sub>2</sub> -UTP	ATP	1%
2'-β-ala-NH <sub>2</sub> -UTP	ATP	3%
2'-C-allyl-UTP	ATP	2%
2'-O-NH <sub>2</sub> -UTP	ATP	1%
2'-O-MTM-UTP	ATP	64%
2'-NH <sub>2</sub> -ATP	GTP	1%
2'-O-MTM-ATP	GTP	1%
2'-NH <sub>2</sub> -CTP	GTP	59%
2'-dCTP	GTP	40%

Table Xa: Incorporation of 2'-bis-UTP and Modified CTP's

modification	2'-bis-UTP	rUTP
CTP	16.1	100
2'-amino-CTP	<b>9.5*</b>	232.7
2'-deoxy-CTP	<b>9.6*</b>	130.1
2'-OMe-CTP	1.9	6.2
2'-MTM-CTP	5.9	5.1
control	1.2	

5

Table Xb: Incorporation of 2'-bis-UTP, 2-amino CTP, and Modified ATP's

modification	2'-bis-UTP and 2'-amino-CTP	rUTP and rCTP
ATP	15.7	100
2'-amino-ATP	2.4	28.9
2'-deoxy-ATP	2.3	146.3
2'-OMe-ATP	2.7	15
2'-F-ATP	4	222.6
2'-MTM-ATP	4.7	15.3
2'-OMe-DAP	1.9	5.7
2'-amino-DAP	<b>8.9*</b>	9.6

10 Numbers shown are a percentage of incorporation compared to the all-RNA control

\* -Bold number indicates best observed rate of modified nucleotide triphosphate incorporation

**Table XI. INCORPORATION OF 2'-his-UTP, 2'-NH<sub>2</sub>-CTP, 2'-NH<sub>2</sub>-DAP, and rGTP USING VARIOUS REACTION CONDITIONS**

Conditions	compared to all rNTP
7	8.7*
8	7*
9	2.3
10	2.7
11	1.6
12	2.5

5 Numbers shown are a percentage of incorporation compared to the all-RNA control

\* Two highest levels of incorporation contained both methanol and LiCl

Table XII: Human C-raf Hammerhead Ribozyme and Target Sequences

nt position	Target Site	SEQ ID. No.	Ribozyme Sequence	SEQ ID. No.
17	GACCGCCUC CCGCUCCC	1	GGGAGCGG CUGAUGAG X CGAA AGGCAGUC	502
23	CUCCCGCUC CCUCACCC	2	GGGUGAGG CUGAUGAG X CGAA AGCGGGAG	503
27	CGCUCCCCUC ACCCGCCG	3	CGGCAGGGU CUGAUGAG X CGAA AGGGAGCG	504
82	CAGGACGUC GGGGCGGC	4	GCCGCCCC CUGAUGAG X CGAA ACGUCCUG	505
97	GCCUGGCU CUCAGGU	5	ACCUGAGG CUGAUGAG X CGAA AGCCAGGC	506
101	GGCUCCCCUC AGGUUUAA	6	UUAAAACCU CUGAUGAG X CGAA AGGGAGCC	507
106	CCUCAGGUU UAAGAAAU	7	AAUUCUUA CUGAUGAG X CGAA ACCUGAGG	508
107	CUCAGGUUU AAGAAUUG	8	CAAUUCUU CUGAUGAG X CGAA AACCUUGAG	509
108	UCAGGUUUU AGAAUUGU	9	ACAAUUCU CUGAUGAG X CGAA AAACCUUGA	510
114	UUAAGAAUU GUUUAAGC	10	GCUUAAAC CUGAUGAG X CGAA AUUCUUA	511
117	AGAAUUGUU UAAGCUGC	11	GCAGCUUA CUGAUGAG X CGAA ACAAUUCU	512
118	GAAUUGUUU AAGCUGCA	12	UGCAGCUU CUGAUGAG X CGAA AACAAUUC	513
119	AAUUGUUUA AGCUGCAU	13	AUGCAGCU CUGAUGAG X CGAA AAACAAUU	514
128	AGCUGCAUC AAUGGAGC	14	GCUCCAUU CUGAUGAG X CGAA AUGCAGCU	515
141	GAGCACAU AAGGGAGC	15	GCUCCUG CUGAUGAG X CGAA AUGUGCUC	516
151	AGGGAGCUU GGAAGACG	16	CGUCUUCU CUGAUGAG X CGAA AGCUCCU	517
162	AAGACGAUC AGCAAUGG	17	CCAUUGCU CUGAUGAG X CGAA AUCGUCUU	518
172	GCAAUUGGUU UGGGAUUC	18	GAAUCCAU CUGAUGAG X CGAA ACCAUUGC	519
173	CAAUGGUUU UGGGAUCA	19	UGAAUCCA CUGAUGAG X CGAA AACCAUUG	520
174	AAUUGGUUUU GGAAUCAA	20	UUGAAUCC CUGAUGAG X CGAA AAACCAUU	521
179	UUUUGGAUU CAAAGAUG	21	CAUCUUG CUGAUGAG X CGAA AUCCAAA	522
180	UUUUGGAUUC AAAGAUGC	22	GCAUCUUU CUGAUGAG X CGAA AAUCCAAA	523
194	UGCCGUGUU UGAUGGCU	23	AGCCAUCU CUGAUGAG X CGAA ACACGGCA	524
195	GCCGUGUUU GAUGGCUC	24	GAGCCAUC CUGAUGAG X CGAA AACACGGC	525
203	UGAUGGCUC CAGCUGCA	25	UGCAGCUG CUGAUGAG X CGAA AGCCAUC	526
213	AGCUGCAUC UCUCUUA	26	GUAGGAGA CUGAUGAG X CGAA AUGCAGCU	527
215	CUGCAUCU CUCUACAA	27	UUGUAGGA CUGAUGAG X CGAA AGAUGCAG	528
217	GCAUCUCU CUACAAUA	28	UAUUGUAG CUGAUGAG X CGAA AGAGAUGC	529
220	UCUCUCCUA CAAUAGUU	29	AAUCUUAUG CUGAUGAG X CGAA AGGGAGA	530
225	CCUACAAUA GUUCAGCA	30	UGCUGAAC CUGAUGAG X CGAA AUUGUAGG	531
228	ACAAUAGUU CAGCAGUU	31	AACUGCUG CUGAUGAG X CGAA ACUAAUGU	532
229	CAAUAGUUC ACCAGUUU	32	AAACUGCU CUGAUGAG X CGAA AACUAUUG	533
236	UCAGCAGUU UGGCUAUC	33	GAUAGCCA CUGAUGAG X CGAA ACUGCUGA	534
237	CAGCAGUUU GGCUAUCA	34	UGAUAGCC CUGAUGAG X CGAA AACUGCUG	535
242	GUUUGGCUA UCAGGCC	35	GGCGCGUA CUGAUGAG X CGAA AGCCAAAC	536
244	UUGGCCAUUC AGGCCCGG	36	CCGGCGCU CUGAUGAG X CGAA AUAGCCAA	537
257	CCGGGCAUC AGAUGAUG	37	CAUCAUCU CUGAUGAG X CGAA AUGCCGG	538
273	GGCAAACUC ACAGAUCC	38	GGAUCUGU CUGAUGAG X CGAA AGUUUGCC	539
280	UCACAGAUC CUUCUAA	39	CUUAGAAG CUGAUGAG X CGAA AUCUGUGA	540
283	CAGAUCCUU CUAAGACA	40	UGUCUUAG CUGAUGAG X CGAA AGGAUCUG	541
284	AGAUCCUUC UAAGACAA	41	UUGUCUUA CUGAUGAG X CGAA AAGGAUCU	542
286	AUCCUUCUA AGACAAGC	42	GCUUGUCU CUGAUGAG X CGAA AGAAGGAU	543
301	GCAACACUA UCCGUGUU	43	AACACCGA CUGAUGAG X CGAA AGUGUUGC	544
303	AACACUAUC CGUGUUUU	44	AAAACACG CUGAUGAG X CGAA AUAGGUU	545
309	AUCCGUGUU UUCUUGCC	45	GGCAAGAA CUGAUGAG X CGAA ACACGGAU	546
310	UCCGUGUUU UCUUUGCC	46	CGGCAAGA CUGAUGAG X CGAA AACACGGA	547
311	CCGUGUUUU CUUGCCGA	47	UCGGCAAG CUGAUGAG X CGAA AAACACGG	548
312	CGUGUUUUUC UUGCCGAA	48	UUCGGCAA CUGAUGAG X CGAA AAAACACG	549
314	UGUUUUUCUU GCCGAACA	49	UGUUCGGC CUGAUGAG X CGAA AGAAAACA	550
339	ACAGUGGUC AAUGUGCG	50	CGCACAUU CUGAUGAG X CGAA ACCACUGU	551
362	AAUGAGCUU GCAUGACU	51	AGUCAUGC CUGAUGAG X CGAA AGCUCAUU	552
375	GACUGCCUU AUGAAAGC	52	GCUUUCAU CUGAUGAG X CGAA AGGCAGUC	553
376	ACUGCCUUA UGAAAGCA	53	UGCUUUCU CUGAUGAG X CGAA AAGGCAGU	554
387	AAAGCACUC AAGGUGAG	54	CUCACCUU CUGAUGAG X CGAA AGUGCUUU	555
425	UGCAGUGUU CAGACUUC	55	GAAGUCUG CUGAUGAG X CGAA ACACUGCA	556
426	GCAGUGUUC AGACUUCU	56	AGAAGUCU CUGAUGAG X CGAA AACACUGC	557
432	UUCAGACUU CUCCACGA	57	UCGUGGAG CUGAUGAG X CGAA AGUCUGAA	558

nt position	Target Site	SEQ ID. No.	Ribozyme Sequence	SEQ ID. No.
433	UCAGACUUC UCCACGAA	58	UUCGUGGA CUGAUGAG X CGAA AAGUCUGA	559
435	AGACUUCUC CACGAACA	59	UGUUCGUG CUGAUGAG X CGAA AGAAGUCU	560
451	ACAAAGGUA AAAAAGCA	60	UGCUUUUU CUGAUGAG X CGAA ACCUUUGU	561
464	AGCACGCUU AGAUUGGA	61	UCCAAUCU CUGAUGAG X CGAA AGCGUGCU	562
465	GCACGCUUA GAUUGGAA	62	UUCCAAUC CUGAUGAG X CGAA AAGCGUGC	563
469	CCUUAGAUU GGAAUACU	63	AGUAUUCG CUGAUGAG X CGAA AUCUAAGC	564
475	AUUGGAAUA CUGAUGCU	64	AGCAUCAG CUGAUGAG X CGAA AUUCCAAU	565
488	UGCUGCGUC UUUGAUUG	65	CAAUAAA CUGAUGAG X CGAA ACGCAGCA	566
490	CUGCGUCUU UGAUUGGA	66	UCCAAUCA CUGAUGAG X CGAA AGACGCAG	567
491	UGCGUCUUU GAUUGGAG	67	CUCCAAUC CUGAUGAG X CGAA AAGACGCA	568
495	UCUUUGAUU GGAGAAGA	68	UCUUCUCC CUGAUGAG X CGAA AUCAAAGA	569
507	GAAGAACUU CAAGUAGA	69	UCUACUUG CUGAUGAG X CGAA AGUUCUUC	570
508	AAGAACUUC AAGUAGAU	70	AUCUACUU CUGAUGAG X CGAA AAGUUCUU	571
513	CUUCAAGUA GAUUUCU	71	AGGAAUAC CUGAUGAG X CGAA ACUUGAAG	572
517	AAGUAGAUU UCCUGGAU	72	AUCCAGGA CUGAUGAG X CGAA AUCUACUU	573
518	AGUAGAUUU CCUGGAUC	73	GAUCCAGG CUGAUGAG X CGAA AAUCUACU	574
519	GUAGAUUUC CUGGAUCA	74	UGAUCCAG CUGAUGAG X CGAA AAAUCUAC	575
526	UCCUGGAUC AUGUUCCC	75	GGGAACAU CUGAUGAG X CGAA AUCCAGGA	576
531	GAUCAUGU CCCUCAC	76	GUGAGGGG CUGAUGAG X CGAA ACAUGAUC	577
532	AUCAUGUUC CCCUCACA	77	UGUGAGGG CUGAUGAG X CGAA AACAUAGAU	578
537	GUUCCCCUC ACAACACA	78	UGUGUUGU CUGAUGAG X CGAA AGGGGAAC	579
551	ACACAACUU UGCUCGGA	79	UCCGAGCA CUGAUGAG X CGAA AGUUGUGU	580
552	CACAACUUU GCUCGGAA	80	UUCCGAGC CUGAUGAG X CGAA AAGLUGUG	581
556	ACUUUGCUC GGAAGACG	81	CGUCUUCC CUGAUGAG X CGAA AGCAAAGU	582
566	GAAGACGUU CCUGAAGC	82	GUUCUAGG CUGAUGAG X CGAA ACGUCUUC	583
567	AAGACGUUC CUGAAGCU	83	AGCUUCAG CUGAUGAG X CGAA AACGUCUU	584
576	CUGAAGCUU GCCUUCUG	84	CAGAAGGC CUGAUGAG X CGAA AGCUUCAG	585
581	CCUUGCCUU CUGUGACA	85	UGUCACAG CUGAUGAG X CGAA AGGCAAGC	586
582	CUUGCCUUC UGUGACAU	86	AUGUCACA CUGAUGAG X CGAA AAGGCAAG	587
591	UGUGACAU CUGACAGAA	87	UUCUGACA CUGAUGAG X CGAA AUGUCACA	588
595	ACAUCUGUC AGAAAUC	88	GAUUUUCU CUGAUGAG X CGAA ACAGAUGU	589
602	UCAGAAAAUU CCUGCUCA	89	UGAGCAGG CUGAUGAG X CGAA AUUUCUGA	590
603	CAGAAAUUC CUGCUCAA	90	UUGAGCAG CUGAUGAG X CGAA AAUUCUG	591
609	UCCUGCUC AAUGGAU	91	AAUCCAUU CUGAUGAG X CGAA AGCAGGAA	592
617	CAAUGGAUU UCGAUGUC	92	GACAUCGA CUGAUGAG X CGAA AUCCAUG	593
618	AAUGGAUUU CGAUGUCA	93	UGACAUCA CUGAUGAG X CGAA AAUCCAUU	594
619	AUGGAUUC GAUGUCAG	94	CUGACAU CUGAUGAG X CGAA AAAUCCAU	595
625	UUCGAUGUC AGACUUGU	95	ACAAGUCU CUGAUGAG X CGAA ACAUCGAA	596
631	GUCAGACUU GUGGUAC	96	GUAGCCAC CUGAUGAG X CGAA AGUCUGAC	597
638	UUGUGGCUA CAAAUUC	97	GAUUUUG CUGAUGAG X CGAA AGCCACAA	598
644	CUACAAAUU UCAUGAGC	98	GCUCUAGA CUGAUGAG X CGAA AAUUGUAG	599
645	UACAAAUUU CAUGAGCA	99	UGCUCUAG CUGAUGAG X CGAA AAUUUGUA	600
646	ACAAAUUUC AUGAGCAC	100	GUGCUCAU CUGAUGAG X CGAA AAAUUUGU	601
658	AGCACUGUA GCACCAA	101	UUUGGUGC CUGAUGAG X CGAA ACAGUGCU	602
669	ACCAAAGUA CCUACUAU	102	AUAGUAGG CUGAUGAG X CGAA ACUUUGGU	603
673	AAGUACCUA CUAUGUGU	103	ACACAUAG CUGAUGAG X CGAA AGGUACUU	604
676	UACCUACUA UGUGUGUG	104	CACACACA CUGAUGAG X CGAA AGUAGGUA	605
694	ACUGGAGUA ACAUCAGA	105	UCUGAUGU CUGAUGAG X CGAA ACUCCAGU	606
699	AGUAACAU CAGACAACU	106	AGUUGUCU CUGAUGAG X CGAA AUGUUACU	607
708	AGACAACUC UUAUUGUU	107	AACAAUAA CUGAUGAG X CGAA AGUUGUCU	608
710	ACAACUCUU AUUGUUUC	108	GAAACAAU CUGAUGAG X CGAA AGAGUUGU	609
711	CAACUCUUA UUGUUUCC	109	GGAAACAA CUGAUGAG X CGAA AAGAGUUG	610
713	ACUCUUAUU GUUUCCAA	110	UUGGAAAC CUGAUGAG X CGAA AAUAGAGU	611
716	CUUAUJGUU UCCAAAUU	111	AAUUUGGA CUGAUGAG X CGAA ACAAAUAG	612
717	UUAUUGUUU CCAAAUUC	112	GAUUUUGG CUGAUGAG X CGAA AACAAUAA	613
718	UAUUGUUUUC CAAAUUCC	113	GGAAUUUG CUGAUGAG X CGAA AAACAAUA	614
724	UCCAAAUU CCACUAAU	114	AAUAGUGG CUGAUGAG X CGAA AUUUGGAA	615
725	UCCAAAUUC CACUAAUG	115	CAAUAGUG CUGAUGAG X CGAA AAUUUGGA	616
730	AUUCCACUA UGGUGAU	116	AUCACCAA CUGAUGAG X CGAA AGUGGAU	617

nt position	Target Site	SEQ ID. No.	Ribozyme Sequence	SEQ ID. No.
732	UCCACUAUU GGUGAUAG	117	CUAUCACC CUGAUGAG X CGAA AUAGUGGA	618
739	UUGGUGUAU GUGGAGUC	118	GACUCCAC CUGAUGAG X CGAA AUCACCAA	619
747	AGUGGAGUC CCAGCACU	119	AGUGCUCC CUGAUGAG X CGAA ACUCCACU	620
756	CCAGCACUA CCUUCUUU	120	AAAGAAGG CUGAUGAG X CGAA AGUGCUCC	621
760	CACUACCUU CUUUGACU	121	AGUCAAAG CUGAUGAG X CGAA AGGUAGUG	622
761	ACUACCUUC UUUGACUA	122	UAGUAAA CUGAUGAG X CGAA AAGGUAGU	623
763	UACCUUCUU UGACUAUG	123	CAUAGUCA CUGAUGAG X CGAA AGAAGGUA	624
764	ACCUUCUUU GACUAUGC	124	GCAUAGUC CUGAUGAG X CGAA AAGAAGGU	625
769	CUUUGACUA UGCGUCGU	125	ACGACGCA CUGAUGAG X CGAA AGUCAAAG	626
775	CUAUGCUC GUAUGCGA	126	UCGCAUAC CUGAUGAG X CGAA ACGCAUAG	627
778	UGCGUCGUA UGCGAGAG	127	CUCUCGCA CUGAUGAG X CGAA ACGACGCA	628
788	GCGAGAGUC UGUUUCCA	128	UGGAAACA CUGAUGAG X CGAA ACUCUCGC	629
792	GAGUCUGUU UCCAGGAU	129	AUCCUGGA CUGAUGAG X CGAA ACAGACUC	630
793	AGUCUGUUU CCAGGAUG	130	CAUCCUGG CUGAUGAG X CGAA AACAGACU	631
794	GUCGUUUUC CAGGAUGC	131	GCAUCCUG CUGAUGAG X CGAA AAACAGAC	632
807	AUGCCUGUU AGUUCUCA	132	UGAGAACU CUGAUGAG X CGAA ACAGGCAU	633
808	UGCCUGUUA GUUCUCAG	133	CUGAGAAC CUGAUGAG X CGAA AACAGGCA	634
811	CUGUUAGUU CUCAGCAC	134	GUGCUGAG CUGAUGAG X CGAA ACUAAACAG	635
812	UGUUAGUUC UCAGCACA	135	UGUGCUGA CUGAUGAG X CGAA AACUAACA	636
814	UUAGUUCUC AGCACAGA	136	UCUGUGCU CUGAUGAG X CGAA AGAACUAA	637
824	GCACAGAUU UUCUACAC	137	GUGUAGAA CUGAUGAG X CGAA AUCUGUGC	638
826	ACAGAUUUA CUACACCU	138	AGGUGUAG CUGAUGAG X CGAA AUAUCUGU	639
827	CAGAUUUC UACACCUC	139	GAGGUGUA CUGAUGAG X CGAA AAUAUCUG	640
829	GAUAUUCUA CACCUCAC	140	GUGAGGUG CUGAUGAG X CGAA AGAAUAUC	641
835	CUACACCUC ACGCCUUC	141	GAAGGCGU CUGAUGAG X CGAA AGGUGUAG	642
842	UCACGCCUU CACCUUUA	142	UAAAGGUG CUGAUGAG X CGAA AGGCGUGA	643
843	CACGCCUUC ACCUUUAA	143	UAAAAGGU CUGAUGAG X CGAA AAGGCGUG	644
848	CUUCACCUU UAACACCU	144	AGGUGUUA CUGAUGAG X CGAA AGGUGAAG	645
849	UUCACCUU AACACCUC	145	GAGGUGUU CUGAUGAG X CGAA AAGGUGAA	646
850	UCACCUUUA ACACCUUC	146	GGAGGUGU CUGAUGAG X CGAA AAAGGUGA	647
857	UAACACCU CAGUCCCU	147	AGGGACUG CUGAUGAG X CGAA AGGUGUUA	648
862	CCUCCAGUC CCUCAUC	148	AGAUGAGG CUGAUGAG X CGAA ACUGGAGG	649
866	CAGUCCUC AUCUGAAG	149	CUUCAGAU CUGAUGAG X CGAA AGGGACUG	650
869	UCCCUCAUC UGAAGGUU	150	AACCUUCA CUGAUGAG X CGAA AUGAGGGA	651
877	CUGAAGGUU CCCUCUCC	151	GGAGAGGG CUGAUGAG X CGAA ACCUUACAG	652
878	UGAAGGUUC CCUCUCCC	152	GGGGAGGG CUGAUGAG X CGAA ACCUUCA	653
882	GGUUCCCCUC UCCCAGAG	153	CUCUGGGA CUGAUGAG X CGAA AGGGAAACC	654
884	UCCCCUCUC CCAGAGGC	154	GCCUCUGG CUGAUGAG X CGAA AGAGGGAA	655
899	GCAGAGGUC GACAUCCA	155	UGGAUGUC CUGAUGAG X CGAA ACCUCUGC	656
905	GUCCGACAUC CACACCUA	156	UAGGUGUG CUGAUGAG X CGAA AUGUGGAC	657
913	CCACACCUA AUGUCCAC	157	GUGGACAU CUGAUGAG X CGAA AGGUGUAG	658
918	CCUAAUGUC CACAUUGU	158	ACCAUGUG CUGAUGAG X CGAA ACAUUAGG	659
927	CACAUUGUC AGCACAC	159	GUGGUGCU CUGAUGAG X CGAA ACCAUGUG	660
960	AGGAUGAUU GAGGAUGC	160	GCAUCCUC CUGAUGAG X CGAA AUCAUCCU	661
972	GAUGCAAUU CGAAGUCA	161	UGACUUCG CUGAUGAG X CGAA AUUGCAUC	662
973	AUGCAAAUC GAAGUCAC	162	GUGACUUC CUGAUGAG X CGAA AAUUGCAU	663
979	UUCGAAGUC ACAGCGAA	163	UUCGCCU CUGAUGAG X CGAA ACUUCGAA	664
989	CAGCGAAUC AGCCUCAC	164	GUGAGGCU CUGAUGAG X CGAA AUUCGCUG	665
995	AUCAGCCUC ACCUUACAG	165	CUGAAGGU CUGAUGAG X CGAA AGGCUGAU	666
1000	CCUCACCUU CAGCCUG	166	CAGGGCUG CUGAUGAG X CGAA AGGUGAGG	667
1001	CUCACCUUC AGCCCUGU	167	ACAGGGCU CUGAUGAG X CGAA AAGGUGAG	668
1010	AGCCCUGUC CAGUAGCC	168	GGCUACUG CUGAUGAG X CGAA ACAGGGCU	669
1015	UGUCCAGUA GCCCCAAC	169	GUUGGGGC CUGAUGAG X CGAA ACUGGACA	670
1027	CCAACAAUC UGAGCCCA	170	UGGGCUCU CUGAUGAG X CGAA AUUGUUGG	671
1046	AGGCUGGGUC ACAGCGGA	171	UCGGGCUGU CUGAUGAG X CGAA ACCAGCCU	672
1092	GCACCAAGUA UCUGGGAC	172	GUCCCCAGA CUGAUGAG X CGAA ACUGGUGC	673
1094	ACCAGUAUC UGGGACCC	173	GGGUCCCCA CUGAUGAG X CGAA AUACUGGU	674
1119	AACAAAAAUU AGGCCUCG	174	CGAGGGCU CUGAUGAG X CGAA AUUUUGUU	675
1120	ACAAAAUUA GGCCUCGU	175	ACGAGGCC CUGAUGAG X CGAA AUUUUGUG	676

nt position	Target Site	SEQ ID. No.	Ribozyme Sequence	SEQ ID. No.
1126	UUAGGCCUC GUGGACAG	176	CUGUCCAC CUGAUGAG X CGAA AGGCCUAA	677
1141	AGAGAGAUU CAAGCUAU	177	AUAGCUUG CUGAUGAG X CGAA AUCUCUCU	678
1142	GAGAGAUUC AAGCUAUU	178	AAUAGCUU CUGAUGAG X CGAA AAUCUCUC	679
1148	JUCAAGCUA UUAUUGGG	179	CCCAAUAA CUGAUGAG X CGAA AGCUUGAA	680
1150	CAAGCUAUU AUUGGGAA	180	UUCCCAAU CUGAUGAG X CGAA AUAGCUUG	681
1151	AAGCUAUUA UUGGGAAA	181	UUUCCCAA CUGAUGAG X CGAA AAUAGCUU	682
1153	CCUAUUAAA GGGAAAUA	182	UAUUUCCC CUGAUGAG X CGAA AAUAAUGC	683
1161	UGGGAAAUA GAAGCCAG	183	CUGGCUUC CUGAUGAG X CGAA AUUUCCCA	684
1184	GAUGCUGUC CACUCGGA	184	UCCGAGUG CUGAUGAG X CGAA ACAGCAUC	685
1189	UGUCCACUC GGAUUGGG	185	CCCAAUCC CUGAUGAG X CGAA AGUGGACA	686
1194	ACUCGGAUU GGGUCAGG	186	CCUGACCC CUGAUGAG X CGAA AUCCGAGU	687
1199	GAUUGGGUC AGGCUCUU	187	AAGAGCCU CUGAUGAG X CGAA ACCCAAUC	688
1205	GUCAGGCUC UUUUGGAA	188	UUCCAAAA CUGAUGAG X CGAA AGCCUGAC	689
1207	CAGGCUCUU UUGGAACU	189	AGUUCCAA CUGAUGAG X CGAA AGAGCCUG	690
1208	AGGCUCUUU UGGAACUG	190	CAGUUCCA CUGAUGAG X CGAA AAGAGCCU	691
1209	GGCUCUUUU GGAACUGU	191	ACAGUUCC CUGAUGAG X CGAA AAAGAGCC	692
1218	GGAACUGUU UAUAGGG	192	CCCUAUAA CUGAUGAG X CGAA ACAGUUCC	693
1219	GAACUGUUU AUAGGGU	193	ACCCUUAAU CUGAUGAG X CGAA AACAGUUC	694
1220	AACUGUUUA UAAGGGUA	194	UACCCUUA CUGAUGAG X CGAA AAACAGUU	695
1222	CUGUUUAAA AGGGAAA	195	UUUACCCU CUGAUGAG X CGAA AAUAAACAG	696
1228	AUAAGGGUA AAUGGCAC	196	GUGCCAUU CUGAUGAG X CGAA ACCCUUAA	697
1245	GGAGAUGUU GCAGUAAA	197	UUUACUGC CUGAUGAG X CGAA ACAUCUCC	698
1251	GUUGCAGUA AAGAUCCU	198	AGGAUCUU CUGAUGAG X CGAA ACUGCAAC	699
1257	GUAAAAGAUC CUAAAGGU	199	ACCUUUAAG CUGAUGAG X CGAA AUCUUUAC	700
1260	AAGAUCCUA AAGGUUGU	200	ACAACCUU CUGAUGAG X CGAA AGGAUCUU	701
1266	CUAAAGGUU GUCGACCC	201	GGGUCGAC CUGAUGAG X CGAA ACCUUUAG	702
1269	AAGGUUGUC GACCCAC	202	GUUGGGUC CUGAUGAG X CGAA ACAACCUU	703
1289	AGAGCAAUU CCAGGCCU	203	AGGCCUGG CUGAUGAG X CGAA AUUGCUCU	704
1290	GAGCAAUUC CAGGCCUU	204	AAGGCCUG CUGAUGAG X CGAA AAUUGCUC	705
1298	CCAGGCCUU CAGGAAUG	205	CAUUCCUG CUGAUGAG X CGAA AGGCCUGG	706
1299	CAGGCCUUC AGGAAUGA	206	UCAUCCU CUGAUGAG X CGAA AAGGCCUG	707
1317	GUGGCUGUU CUGCGCAA	207	UUGCGCAG CUGAUGAG X CGAA ACAGCCAC	708
1318	UGGCUGUUC UGCGCAA	208	UUUGCGCA CUGAUGAG X CGAA AACAGCCA	709
1344	GUGAACAUU CUGCUUUU	209	AAAAGCAG CUGAUGAG X CGAA AUGUUCAC	710
1345	UGAACAUUC UGCUUUUC	210	GAAAAGCA CUGAUGAG X CGAA AAUGUUCA	711
1350	AUUCUGCUU UUCAUGGG	211	CCCAUGAA CUGAUGAG X CGAA AGCAGAAU	712
1351	UUCUGCUUU UCAUGGGG	212	CCCCAUGA CUGAUGAG X CGAA AAGCAGAA	713
1352	UCUGCUUUU CAUGGGGU	213	ACCCCAUG CUGAUGAG X CGAA AAAGCAGA	714
1353	CUGCUUUUC AUUGGGUA	214	UACCCCCAU CUGAUGAG X CGAA AAAAGCAG	715
1361	CAUGGGGUU CAUGACAA	215	UUGUCAUG CUGAUGAG X CGAA ACCCCAU	716
1386	CUGGCAAUU GUGACCCA	216	UGGGUCAC CUGAUGAG X CGAA AUUGCCAG	717
1416	AGCAGCCUC UACAAACA	217	UGUUUGUA CUGAUGAG X CGAA AGGCUGCU	718
1418	CAGCCUCUA CAAACACC	218	GGUGUUUG CUGAUGAG X CGAA AGAGGCUG	719
1434	CUGCAUGUC CAGGAGAC	219	GUCUCCUG CUGAUGAG X CGAA ACAUGCAG	720
1448	GACCAAGUU UCAGAUGU	220	ACAUCUGA CUGAUGAG X CGAA ACUUGGUC	721
1449	ACCAAGUUU CAGAUGUU	221	AAACAUCUG CUGAUGAG X CGAA AACUUGGU	722
1450	CCAAGUUUC AGAUGUUC	222	GAACAUU CUGAUGAG X CGAA AAACUUGG	723
1457	UCAGAUGUU CCAGCUAA	223	UUAGCUGG CUGAUGAG X CGAA ACAUCUGA	724
1458	CAGAUGUUC CAGCUAAU	224	AUUAGCUG CUGAUGAG X CGAA AACAUUCG	725
1464	UUCCAGCUA AUUGACAU	225	AUGUCAAU CUGAUGAG X CGAA AGCUGGAA	726
1467	CAGCUAAU GACAUUGC	226	GCAAUGUC CUGAUGAG X CGAA AUUAGCUG	727
1473	AUUGACAUU GCCCGGCA	227	UGCCGGGC CUGAUGAG X CGAA AUGUCAAU	728
1489	AGACGGCUC AGGGAAUG	228	CAUUCCCU CUGAUGAG X CGAA AGCCGUU	729
1502	AAUGGACUA UUUGCAUG	229	CAUGCAAA CUGAUGAG X CGAA AGUCCAUU	730
1504	UGGACUAUU UGCAUGCA	230	UGCAUGCA CUGAUGAG X CGAA AUAGUCCA	731
1505	GGACUAUUU GCAUGCAA	231	UUGCAUGC CUGAUGAG X CGAA AAUAGUCC	732
1521	AAGAACAUUC AUCCAUAG	232	CUAUGGAU CUGAUGAG X CGAA AUGUUCUU	733
1524	AAACAUCAUC CAUAGAGA	233	UCUCUAUG CUGAUGAG X CGAA AUGAUGUU	734
1528	UCAUCCAU AAGACAU	234	CAUGUCUC CUGAUGAG X CGAA AUGGAUGA	735

nt position	Target Site	SEQ ID. No.	Ribozyme Sequence	SEQ ID. No.
1541	CAUGAAUC CAACAAUA	235	UAUUGUUG CUGAUGAG X CGAA AUUUCAG	736
1549	CCAACAAUA UAUUUCUC	236	GAGAAUA CUGAUGAG X CGAA AUUGUUGG	737
1551	AACAAUUA UUUCUCCA	237	UGGAGAAA CUGAUGAG X CGAA AUAUUGU	738
1553	CAAUUAUUA UCUCCAUG	238	CAUGGAGA CUGAUGAG X CGAA AUUAUUG	739
1554	AAUUAUAAA CUCCAUGA	239	UCAUGGAG CUGAUGAG X CGAA AAUUAUU	740
1555	AUAAUAAAUC UCCAUGAA	240	UUCAUGGA CUGAUGAG X CGAA AAAUUAU	741
1557	AUAAUUCUC CAUGAAGG	241	CCUUCAUG CUGAUGAG X CGAA AGAAAUAU	742
1568	UGAAGGCUU AACAGUGA	242	UCACUGUU CUGAUGAG X CGAA AGCCUUC	743
1569	GAAGGCCUA ACAGUGAA	243	UUCACUGU CUGAUGAG X CGAA AAGCCUUC	744
1581	GUGAAAAAU GGAGAUU	244	AAAUCUCC CUGAUGAG X CGAA AUUUUCAC	745
1588	UUGGAGAUU UGGUUUUG	245	CAAACCAA CUGAUGAG X CGAA AUCUCCAA	746
1589	UGGAGAUUU UGGUUUUGG	246	CCAAACCA CUGAUGAG X CGAA AAUCUCCA	747
1590	GGAGAUUUU GGUUUGGC	247	GCCAAACC CUGAUGAG X CGAA AAAUCUCC	748
1594	AUUUUGGUU UGGCAACA	248	UGUUGGCC CUGAUGAG X CGAA ACCAAAAAU	749
1595	UUUUGGUUU GGCAACAG	249	CUGUUGCC CUGAUGAG X CGAA AACCAAAA	750
1605	GCAACAGUA AAGUCACG	250	CGUGACUU CUGAUGAG X CGAA ACUGUUGC	751
1610	AGUAAAGUC ACGCUGGA	251	UCCAGCGU CUGAUGAG X CGAA ACUUUACU	752
1624	GGAGUGGUU CUCAGCAG	252	CUGCUGAG CUGAUGAG X CGAA ACCACUCC	753
1625	GAGUGGUUC UCAGCAGG	253	CCUGCUGA CUGAUGAG X CGAA AACCACUC	754
1627	GUGGUUCUC AGCAGGUU	254	AACCUGCU CUGAUGAG X CGAA AGAACCCAC	755
1635	CAGCAGGUU GAACAACC	255	GGUUGUUC CUGAUGAG X CGAA ACCUGCUG	756
1645	AACAACCUA CUGGCUCU	256	AGAGCCAG CUGAUGAG X CGAA AGGUUGUU	757
1652	UACUGGCUC UGUCCUCU	257	AGAGGACA CUGAUGAG X CGAA AGCCAGUA	758
1656	GGCUCUGUC CUCUGGAU	258	AUCCAGAG CUGAUGAG X CGAA ACAGAGCC	759
1659	UCUGUCCUC UGGAUGGC	259	GCCAUCCA CUGAUGAG X CGAA AGGACAGA	760
1680	GAGGUGAU CGAAUGCA	260	UGCAUUCG CUGAUGAG X CGAA AUCACCUC	761
1693	UGCAGGAUA ACAACCCA	261	UGGGGUUGU CUGAUGAG X CGAA AUCCUGCA	762
1703	CAACCCAUU CAGUUUCC	262	GGAAACUG CUGAUGAG X CGAA AUGGGUUG	763
1704	AACCCAUUC AGUUUCCA	263	UGGAAACU CUGAUGAG X CGAA AAUGGGUU	764
1708	CAUUCAGUU UCCAGUGG	264	CGACUGGA CUGAUGAG X CGAA ACUGAAUG	765
1709	AUUCAGUUU CCAGUGGG	265	CCGACUGG CUGAUGAG X CGAA AACUGAAU	766
1710	UUCAGUUUC CAGUGGGA	266	UCCGACUG CUGAUGAG X CGAA AAACUGAA	767
1715	UUUCCAGUC GGAUGUCU	267	AGACAUCC CUGAUGAG X CGAA ACUGGAAA	768
1722	UCGGGAUGU UACUCCUA	268	UAGGAGUA CUGAUGAG X CGAA ACAUCCGA	769
1724	GGGAUGCUA CUCCUAUG	269	CAUAGGAG CUGAUGAG X CGAA AGACAUCC	770
1727	UGUCUACUC CUAUGGCA	270	UGCCAUAG CUGAUGAG X CGAA AGUAGACA	771
1730	CUACUCCUA UGGCAUCG	271	CGAUGCCA CUGAUGAG X CGAA AGGAGUAG	772
1737	UAUGGCAUC GUAUUGUA	272	UACAAUAC CUGAUGAG X CGAA AUGCCAU	773
1740	GGCAUCGUA UUGUAUGA	273	UCAUACAA CUGAUGAG X CGAA ACGAUGCC	774
1742	CAUCGUAUU GUUAUAC	274	GUUCAUAC CUGAUGAG X CGAA AUACGAUG	775
1745	CGUAUUGUA UGAACUGA	275	UCAGUUCA CUGAUGAG X CGAA ACAAUACG	776
1767	GGGGAGCUU CCUUAUUC	276	GAUAUAGG CUGAUGAG X CGAA AGCUCUCCC	777
1768	GGGAGCUUC CUUAUUCU	277	AGAAUAG CUGAUGAG X CGAA AAGCUCUCC	778
1771	AGCUUCCUU AUUCUCAC	278	GUGAGAAU CUGAUGAG X CGAA AGGAAGCU	779
1772	GCUUCCUUU UUCUCACA	279	UGUGAGAA CUGAUGAG X CGAA AAGGAAGC	780
1774	UUCCUUUAU CUCACAU	280	GAUGUGAG CUGAUGAG X CGAA AUUAGGAA	781
1775	UCCUUUAUUC UCACAUCA	281	UGAUGUGA CUGAUGAG X CGAA AAAUAGGA	782
1777	CUUAAUCUC ACAUCAAC	282	GUUGAUGU CUGAUGAG X CGAA AGAAUAAG	783
1782	UCUCACAU CAAACACG	283	CGGUUGUU CUGAUGAG X CGAA AUGUGAGA	784
1795	ACCGAGGAUC AGAUCAUC	284	GAUGAUCU CUGAUGAG X CGAA AUCUCGGU	785
1800	GAUCAGAUC AUCUUCAU	285	AUGAAGAU CUGAUGAG X CGAA AUCUGAUC	786
1803	CAGAUCAUC UUCAUGGU	286	ACCAUGAA CUGAUGAG X CGAA AUGAUCUG	787
1805	GAUCAUCUU CAUGGUGG	287	CCACCAUG CUGAUGAG X CGAA AGAUGAUC	788
1806	AUCAUCUUC AUGGUGGG	288	CCCACCAU CUGAUGAG X CGAA AAGAUGAU	789
1823	CCGAGGAUA UGCCUCCC	289	GGGAGGCA CUGAUGAG X CGAA AUCCUCGG	790
1829	AUAUGCCUC CCCAGAUC	290	GAUCUGGG CUGAUGAG X CGAA AGGCAUAU	791
1837	CCCCAGAUC UUAGUAAG	291	CUUACUA CUGAUGAG X CGAA AUCUGGGG	792
1839	CCAGAUCUU AGUAAGCU	292	AGCUUACU CUGAUGAG X CGAA AGAUCUGG	793
1840	CAGAUCUUA GUAAGCUA	293	UAGCUUAC CUGAUGAG X CGAA AAGAUCUG	794

nt position	Target Site	SEQ ID. No.	Ribozyme Sequence	SEQ ID. No.
1843	AUCUUAGUA AGCUAUAU	294	AUAUAGCU CUGAUGAG X CGAA ACUAAGAU	795
1848	AGUAAGCUA UAUAGAA	295	UUCUUUAUA CUGAUGAG X CGAA AGCUUACU	796
1850	UAAGCUUA UAAGAACU	296	AGUUCUUA CUGAUGAG X CGAA AUAGCUUA	797
1852	AGCUAUACU AGAACUGC	297	GCAGUUUCU CUGAUGAG X CGAA AUAUAGCU	798
1884	AGGCUGGUA GCUGACUG	298	CAGUCAGC CUGAUGAG X CGAA ACCAGCCU	799
1905	AAAGAAAGUA AAGGAAGA	299	UCUUCUUU CUGAUGAG X CGAA ACUUUCUU	800
1921	AGAGGCCUC UUUUUCC	300	GGGAAAAA CUGAUGAG X CGAA AGGCCUCU	801
1923	AGGCCUCUU UUUCCCC	301	UGGGGAAA CUGAUGAG X CGAA AGAGGCCU	802
1924	GGCCUCUUU UUCCCCAG	302	CUGGGGAA CUGAUGAG X CGAA AAGAGGCC	803
1925	GCCUCUUUU UCCCCAGA	303	UCUGGGGA CUGAUGAG X CGAA AAAAGAGC	804
1926	CCUCUUUUU CCCCAGAU	304	AUCUGGGG CUGAUGAG X CGAA AAAAGAGG	805
1927	CUCUUUUUC CCCAGAUC	305	GAUCUGGG CUGAUGAG X CGAA AAAAAGAG	806
1935	CCCCAGAUC CUGUCUUC	306	GAAGACAG CUGAUGAG X CGAA AUCUGGG	807
1940	GAUCCUGUC UUCAUUG	307	CAAUGGAA CUGAUGAG X CGAA ACAGGAUC	808
1942	UCCUGUCUU CCAUUGAG	308	CUCAAUUG CUGAUGAG X CGAA AGACAGGA	809
1943	CCUGUCUUC CAUUGAGC	309	GCUCAAUG CUGAUGAG X CGAA AAGACAGG	810
1947	UCUUCCAUU GAGCUGCU	310	AGCAGCUC CUGAUGAG X CGAA AUGGAAGA	811
1956	GAGCUGCUC CAACACUC	311	GAGUGUUG CUGAUGAG X CGAA AGCAGCUC	812
1964	CCAACACUC UCUACCGA	312	UCGGUAGA CUGAUGAG X CGAA AGUGUUGG	813
1966	AAACACUCUC UACCGAAG	313	CUUCGUA CUGAUGAG X CGAA AGAGUUU	814
1968	CACUCUCUA CCGAAGAU	314	AUCUUCGG CUGAUGAG X CGAA AGAGAGUG	815
1977	CCGAAGAUC AACCAGG	315	CUCCGGUU CUGAUGAG X CGAA AUCUUCGG	816
1990	GGAGCGCUU CCGAGCCA	316	UGGCUCUG CUGAUGAG X CGAA AGCGCUCC	817
1991	GAGCGCUUC CGAGCCAU	317	AUGGCUCG CUGAUGAG X CGAA AAGCGCUC	818
2000	CGAGCCAUC CUUGCAUC	318	GAUGCAAG CUGAUGAG X CGAA AUGGCUCG	819
2003	GCCAUCUU GCAUCGGG	319	CCCGAUGC CUGAUGAG X CGAA AGGAUGGC	820
2008	CCUUGCAUC GGGCAGGG	320	GGCUGCCC CUGAUGAG X CGAA AUGCAAGG	821
2029	CUGAGGAUA UCAAUUGC	321	AGCAUUGA CUGAUGAG X CGAA AUCCUCAG	822
2031	GAGGAUAAU AAUGCUUG	322	CAAGCAUU CUGAUGAG X CGAA AUAUCCUC	823
2038	UCAAAUGCUU GCACGCUG	323	CAGCGUGC CUGAUGAG X CGAA AGCAUUGA	824
2054	GACCACGUC CCCGAGGC	324	GCCUCGGG CUGAUGAG X CGAA ACGUGGUC	825
2070	CUGCCUGUC UUCUAGUU	325	AACUAGAA CUGAUGAG X CGAA ACAGGCAG	826
2072	GCCUGUCUU CUAGUUGA	326	UCAACUAG CUGAUGAG X CGAA AGACAGGC	827
2073	CCUGUCUUC UAGUUGAC	327	GUCAACUA CUGAUGAG X CGAA AAGACAGG	828
2075	UGUCUUCUA GUUGACUU	328	AAGUCAAC CUGAUGAG X CGAA AGAAGACA	829
2078	CUUCUAGUU GACUUUGC	329	GCAAAGUC CUGAUGAG X CGAA ACUAGAAG	830
2083	AGUUGACUU UGCACCU	330	CAGGUGCA CUGAUGAG X CGAA AGUCAACU	831
2084	GUUGACUUU GCACCU	331	ACAGGUGC CUGAUGAG X CGAA AAGUCAAC	832
2093	GCACUGUC UUCAGGU	332	AGCCUGAA CUGAUGAG X CGAA ACAGGUGC	833
2095	ACCUGUCUU CAGGCUGC	333	GCAGCCUG CUGAUGAG X CGAA AGACAGGU	834
2096	CCUGUCUUC AGGCUGC	334	GGCAGCCU CUGAUGAG X CGAA AAGACAGG	835
2136	GCACCCACUU UUCUGCUC	335	GAGCAGAA CUGAUGAG X CGAA AGUGGUGC	836
2137	CACCAUUU UCUGCUCC	336	GGAGCAGA CUGAUGAG X CGAA AAGUGGUG	837
2138	ACCACUUUU CUGCUCCC	337	GGGAGCAG CUGAUGAG X CGAA AAAGUGGU	838
2139	CCACUUUUU UGCUCUCC	338	AGGGAGCA CUGAUGAG X CGAA AAAAGUGG	839
2144	UUUCUGCUC CCUUUCUC	339	GAGAAAGG CUGAUGAG X CGAA AGCAGAAA	840
2148	UGCUCUCCUU UCUCCAGA	340	UCUGGAGA CUGAUGAG X CGAA AGGGAGCA	841
2149	GCUCCCCUUU CUCCAGAG	341	CUCUGGAG CUGAUGAG X CGAA AAGGGAGC	842
2150	CUCCCUUUC UCCAGAGG	342	CCUCUGGA CUGAUGAG X CGAA AAAGGGAG	843
2152	CCQUUUCUC CAGAGGCA	343	UGCCUCUG CUGAUGAG X CGAA AGAAAGGG	844
2171	ACACAUUUU UUCAGAGA	344	UCUCUGAA CUGAUGAG X CGAA ACAUGUGU	845
2172	CACAUUUUU UCAAGAGA	345	UUCUCUGA CUGAUGAG X CGAA AACAUUG	846
2173	ACAUGUUUU CAGAGAA	346	CUUUCUG CUGAUGAG X CGAA AAACAUGU	847
2174	CAUGUUUUC AGAGAA	347	GCUUCUCU CUGAUGAG X CGAA AAAACAUG	848
2184	GAGAAGCUC UGCUAAG	348	CCUUAGCA CUGAUGAG X CGAA AGCUUCUC	849
2189	GCUCUGCUA AGGACUU	349	AAGGUCCU CUGAUGAG X CGAA AGCAGAGC	850
2197	AAGGACCUU CUAGACUG	350	CAGCUUAG CUGAUGAG X CGAA AGGUCCUU	851
2198	AGGACCUUC UAGACUG	351	GCAGUCUA CUGAUGAG X CGAA AAGGUCCU	852
2200	GACCUUCUA GACUGCUC	352	GAGCAGUC CUGAUGAG X CGAA AGAAGGUC	853

nt position	Target Site	SEQ ID. No.	Ribozyme Sequence	SEQ ID. No.
2208	AGACUGCUC ACAGGGCC	353	GGCCCUGU CUGAUGAG X CGAA AGCAGUCU	854
2218	CAGGGCCUU AACUUCAU	354	AUGAAGUU CUGAUGAG X CGAA AGGCCCUG	855
2219	AGGGCCUUA ACUUCAUG	355	CAUGAAGU CUGAUGAG X CGAA AAGGCCU	856
2223	CCUUACACUU CAUGUUGC	356	GCAACAU CUGAUGAG X CGAA AGUUAAGG	857
2224	CUUACAUUC AUGUUGCC	357	GGCAACAU CUGAUGAG X CGAA AAGUUAAG	858
2229	CUUCAUGUU GCCUUCU	358	AAGAAGGC CUGAUGAG X CGAA ACAUGAAG	859
2234	UGUUGCCUU CUUUCUA	359	UAGAAAAG CUGAUGAG X CGAA AGGCAACA	860
2235	GUUGCCUUC UUUUCUAU	360	AUAGAAA CUGAUGAG X CGAA AAGGCAAC	861
2237	UGCCUUCUU UUCUAUCC	361	GGAUAGAA CUGAUGAG X CGAA AGAAGGCA	862
2238	GCCUUUCUUU UCUAUCC	362	GGAUAGA CUGAUGAG X CGAA AAGAAGGC	863
2239	CCUUCUUUU CUAUCCCU	363	AGGGAUAG CUGAUGAG X CGAA AAGAAGG	864
2240	CUUCUUUUC UAUCCCUU	364	AAGGGAU CUGAUGAG X CGAA AAAAGAAG	865
2242	UCUUUUUCUA UCCCCUUG	365	CAAAGGGA CUGAUGAG X CGAA AGAAAAGA	866
2244	UUUUUCUAJC CCUUCUGG	366	CCCAAGG CUGAUGAG X CGAA AUAGAAAA	867
2248	CUAUCCCCU UGGGGCCU	367	AGGGCCCA CUGAUGAG X CGAA AGGGAUAG	868
2249	UAUCCCCUU GGGCCUG	368	CAGGGCCC CUGAUGAG X CGAA AAGGGAU	869
2273	GAAGCCAUU UGCAGUGC	369	GCACUGCA CUGAUGAG X CGAA AUGCUUC	870
2274	AAGCCAUUU GCAGUGCU	370	AGCACUGC CUGAUGAG X CGAA AAUGCUU	871
2290	UGGUGUGUC CUGCUCCC	371	GGGAGCAG CUGAUGAG X CGAA ACACACCA	872
2296	GUCCUGCUC CCUCCCCA	372	UGGGGAGG CUGAUGAG X CGAA AGCAGGAC	873
2300	UGCUCCCUC CCCACAUU	373	AAUGUGGG CUGAUGAG X CGAA AGGGAGCA	874
2308	CCCCACAUU CCCCAUGC	374	GCAUGGGG CUGAUGAG X CGAA AUGUGGG	875
2309	CCCACAUUC CCCAUGC	375	AGCAUGGG CUGAUGAG X CGAA AAUGUGGG	876
2318	CCCAUGCUC AAGGCCCA	376	UGGGCCUU CUGAUGAG X CGAA AGCAUGGG	877
2331	CCCAGCCUU CUGUAGAU	377	AUCUACAG CUGAUGAG X CGAA AGGCUGGG	878
2332	CCAGCCUUUC UGUAGAUG	378	CAUCUACA CUGAUGAG X CGAA AAGGCUGG	879
2336	CCUUCUGUA GAUGCGCA	379	UGCGCAUC CUGAUGAG X CGAA ACAGAAGG	880
2354	GUGGAUGUU GAUGGUAG	380	CUACCAUC CUGAUGAG X CGAA ACAUCCAC	881
2361	UUGAUGGUA GUACAAA	381	UUUUGUAC CUGAUGAG X CGAA ACCAUCA	882
2364	AUGGUAGUA CAAAAAGC	382	GCUUUUUG CUGAUGAG X CGAA ACUACAU	883
2393	CCAGCUGUU GGCUACAU	383	AUGUAGCC CUGAUGAG X CGAA ACAGCUGG	884
2398	UGUUGGCUA CAUGAGUA	384	UACUCAUG CUGAUGAG X CGAA AGCCAACA	885
2406	ACAUGAGUA UUUAGAGG	385	CCUCUAAA CUGAUGAG X CGAA ACUCAUG	886
2408	AUGAGUAUU UAGAGGAA	386	UUCCUCUA CUGAUGAG X CGAA AUACUCAU	887
2409	UGAGUAUUU AGAGGAAG	387	CUUCCUCU CUGAUGAG X CGAA AAUACUCA	888
2410	GAGUAUUUU GAGGAAGU	388	ACUUCUC CUGAUGAG X CGAA AAAUACUC	889
2419	GAGGAAGUA AGGUAGCA	389	UGCUACCU CUGAUGAG X CGAA ACUUCUC	890
2424	AGUAAGGUA GCAGGCAG	390	CUGCCUGC CUGAUGAG X CGAA ACCUUCU	891
2434	CAGGCAGUC CAGCCCUG	391	CAGGGCUG CUGAUGAG X CGAA ACUGCCUG	892
2462	CAUGGGAUU UGGAAA	392	AUUUCCAA CUGAUGAG X CGAA AUCCCCAU	893
2463	AUGGGAUUU UGGAAAUC	393	GAUUUCCA CUGAUGAG X CGAA AAUCCCCAU	894
2464	UGGGAUUUU GGAAUCA	394	UGAUUUCC CUGAUGAG X CGAA AAAUCCCCA	895
2471	UGGGAAUUC AGCUUCUG	395	CAGAACU CUGAUGAG X CGAA AUUCCCAA	896
2476	AAUCAGCUU CUGGAGGA	396	UCCUCAG CUGAUGAG X CGAA AGCUGAUU	897
2477	AUCAGCUUC UGGAGGAA	397	UUCCUCCA CUGAUGAG X CGAA AAGCUGAU	898
2493	AUGCAUGUC ACAGGGGG	398	CCGCCUGU CUGAUGAG X CGAA ACAUGCAU	899
2506	GCAGGGACUU UCUUCAGA	399	UCUGAAGA CUGAUGAG X CGAA AGUCCCG	900
2507	CGGGACUUU CUUCAGAG	400	CUCUGAAG CUGAUGAG X CGAA AAGUCCCG	901
2508	GGGACUUUC UUCAGAGA	401	UCUCUGAA CUGAUGAG X CGAA AAAGUCCC	902
2510	GACUUUCUU CAGAGAGU	402	ACUCUCUG CUGAUGAG X CGAA CGAA AGAAAGUC	903
2511	ACUUUCUUC AGAGAGUG	403	CACUCUCU CUGAUGAG X CGAA AAGAAAGU	904
2536	CCAGACAUU UUGCACAU	404	AUGUGCAA CUGAUGAG X CGAA AUGUCUGG	905
2537	CAGACAUUU UGCACAU	405	UAUGUGCA CUGAUGAG X CGAA AAUGUCUG	906
2538	AGACAUUUU GCACAUAA	406	UUAUGUGC CUGAUGAG X CGAA AAAUGUCU	907
2545	UUGCACAUUA AGGCACCA	407	UGGUGCCU CUGAUGAG X CGAA AUGUGCAA	908
2577	CCGAGACUC UGGCCGCC	408	GGCGGCCA CUGAUGAG X CGAA AGUCUCGG	909
2600	AGCCUGCUU UGGUACUA	409	UAGUACCA CUGAUGAG X CGAA AGCAGGCU	910
2601	GCCUGCUUU GGUACAU	410	AUAGUACC CUGAUGAG X CGAA AAGCAGGC	911
2605	GUUUGGUA CUAUGGAA	411	UUCCAUAG CUGAUGAG X CGAA ACCAAAGC	912

nt position	Target Site	SEQ ID. No.	Ribozyme Sequence	SEQ ID. No.
2609	UUGGUACUA UGGAACUU	412	AAGUUCCA CUGAUGAG X CGAA AGUACCAA	913
2616	AUGGAACUU UUCUUAGG	413	CCUAAGAA CUGAUGAG X CGAA AGUCCAU	914
2617	UGGAACUUU UCUUAGGG	414	CCCUAAGA CUGAUGAG X CGAA AAGUCCA	915
2618	GGAACUUUU CUUAGGGG	415	CCCCUAAG CUGAUGAG X CGAA AAAGUCC	916
2619	GAACUUUUC UUAGGGGA	416	UCCCCUAA CUGAUGAG X CGAA AAAAGUUC	917
2621	ACUUUUCUU AGGGGACA	417	UGUCCCCU CUGAUGAG X CGAA AGAAAAGU	918
2622	CUUUUCUUA GGGGACAC	418	GUGUCCCC CUGAUGAG X CGAA AAGAAAAG	919
2633	GGACACGUC CUCCUUUC	419	GAAAGGAG CUGAUGAG X CGAA ACGUGUCC	920
2636	CACGUCCUC CUUUCACA	420	UGUGAAAG CUGAUGAG X CGAA AGGACGUG	921
2639	GUCCUCCUU UCACAGCU	421	AGCUGUGA CUGAUGAG X CGAA AGGAGGAC	922
2640	UCCUCCUUU CACAGCUU	422	AAGCUGUG CUGAUGAG X CGAA AAGGAGGA	923
2641	CCUCCUUUC ACAGCUUC	423	GAAGCUGU CUGAUGAG X CGAA AAAGGAGG	924
2648	UCACAGCUU CUAAGGUG	424	CACCUUAG CUGAUGAG X CGAA AGCUGUGA	925
2649	CACAGCUUC UAAGGUGU	425	ACACCUUA CUGAUGAG X CGAA AAGCUGUG	926
2651	CAGCUUCUA AGGGUCC	426	GGACACCU CUGAUGAG X CGAA AGAACUG	927
2658	UAAGGUGUC CAGUGCAU	427	AUGCACUG CUGAUGAG X CGAA ACACCUUA	928
2667	CAGUGCAUU GGGGAUGGU	428	ACCAUCCC CUGAUGAG X CGAA AUGCACUG	929
2676	GGGAUGGUU UUCCAGGC	429	GCCUGGAA CUGAUGAG X CGAA ACCAUCCC	930
2677	GGAUAGGUUU UCCAGGCA	430	UGCCUGGA CUGAUGAG X CGAA AACCAUCC	931
2678	GAUGGUUUU CCAGGCAA	431	UUGCCUGG CUGAUGAG X CGAA AAACCAUC	932
2679	AUGGUUUUC CAGGCAAG	432	CUUGCCUG CUGAUGAG X CGAA AAAACCAU	933
2693	AAGGCACUC GGCCAAUC	433	GAUUGGCC CUGAUGAG X CGAA AGUGCCU	934
2701	CGGCCAAUC CGCAUCUC	434	GAGAUGCG CUGAUGAG X CGAA AUUGGCCG	935
2707	AUCCGCAUC UCAGGCCU	435	AGGGCUGA CUGAUGAG X CGAA AUGCGAU	936
2709	CCGCAUCUC AGCCCCUC	436	AGAGGGCU CUGAUGAG X CGAA AGAUGCGG	937
2716	UCAGCCCCUC UCAGGAGC	437	GCUCUCUGA CUGAUGAG X CGAA AGGGCUGA	938
2718	AGCCCCUC AGGAGCAG	438	CUGCUCCU CUGAUGAG X CGAA AGAGGGCU	939
2728	GGAGCAGUC UUCCAUCA	439	UGAUGGAA CUGAUGAG X CGAA ACUGCUCC	940
2730	AGCAGUCUU CCAUCAG	440	CAUGAUGG CUGAUGAG X CGAA AGACUGCU	941
2731	GCAGUCUUC CAUCAU	441	GCAUGAUG CUGAUGAG X CGAA AAGACUGC	942
2735	UCUUCCAU AUGCUGAA	442	UUCAGCAU CUGAUGAG X CGAA AUGGAAGA	943
2745	UGCUGAAU UUGUCUUC	443	GAAGACAA CUGAUGAG X CGAA AUUCAGCA	944
2746	GCUGAAUUU UGUCUUCC	444	GGAAGACA CUGAUGAG X CGAA AAUUCAGC	945
2747	CUGAAUUUU GUCUUCCA	445	UGGAAGAC CUGAUGAG X CGAA AAAUUCAG	946
2750	AAUUUUGUC UUCCAGGA	446	UCCUGGAA CUGAUGAG X CGAA ACAAAAUU	947
2752	UUUUGUCUU CCAGGAGC	447	GCUCUCCUG CUGAUGAG X CGAA AGACAAAA	948
2753	UUUGUCUUC CAGGAGCU	448	AGCUCCUG CUGAUGAG X CGAA AAGACAAA	949
2768	CUGCCCCUA UGGGGCGG	449	CCGCCCCA CUGAUGAG X CGAA AGGGCAG	950
2795	CAGCCUGUU UCUCUAAAC	450	GUUAGAGA CUGAUGAG X CGAA ACAGGCUG	951
2796	AGCCUGUUU CUCUAA	451	UGUUUAGAG CUGAUGAG X CGAA AACAGGC	952
2797	GCCUGUUUC UCUUACAA	452	UUGUUAGA CUGAUGAG X CGAA AAACAGGC	953
2799	CUGUUUCUC UAACAAAC	453	GUUUGUUA CUGAUGAG X CGAA AGAAACAG	954
2801	GUUUCUCUA ACAAAACAA	454	UUGUUUGU CUGAUGAG X CGAA AGAGAAAC	955
2825	AACAGCCUU GUUUCUCU	455	AGAGAAAC CUGAUGAG X CGAA AGGCUGUU	956
2828	AGCCUUGUU UCUCUAGU	456	ACUAGAGA CUGAUGAG X CGAA ACAAGGC	957
2829	GCCUUUGUUU CUCUAGUC	457	GACUAGAG CUGAUGAG X CGAA AACAAGGC	958
2830	CCUUGUUUC UCUAGUCA	458	UGACUAGA CUGAUGAG X CGAA AAACAAGG	959
2832	UUGUUUCUC UAGUCACA	459	UGUGACUA CUGAUGAG X CGAA AGAAACAA	960
2834	GUUUCUCUA GUCACAU	460	GAUGUGAC CUGAUGAG X CGAA AGAGAAAC	961
2837	UCYCUAGUC ACAUCAUG	461	CAUGAUGU CUGAUGAG X CGAA ACUAGAGA	962
2842	AGUCACACA AUGUGUAU	462	AUACACAU CUGAUGAG X CGAA AUGUGACU	963
2849	UCAUGUGUA UACAAGGA	463	UCCUUGUA CUGAUGAG X CGAA ACACAU	964
2851	AUGUGUUA CAAGGAAG	464	CUUCCUUG CUGAUGAG X CGAA AUACACAU	965
2868	CCAGGAAUA CAGGUUUU	465	AAAACCUG CUGAUGAG X CGAA AUUCCUGG	966
2874	AUACAGGUU UUCUUGAU	466	AUCAAGAA CUGAUGAG X CGAA ACCUGUAU	967
2875	UACAGGUUU UCUUAGUG	467	CAUCAAGA CUGAUGAG X CGAA AACCU	968
2876	ACAGGUUUU CUUGAUGA	468	UCAUCAAG CUGAUGAG X CGAA AAACCUGU	969
2877	CAGGUUUUC UUGAUGAU	469	AUCAUCAA CUGAUGAG X CGAA AAAACCU	970
2879	GGUUUUCCU GAUGAUUU	470	AAAUCAU CUGAUGAG X CGAA AGAAAACC	971

nt position	Target Site	SEQ ID. No.	Ribozyme Sequence	SEQ ID. No.
2886	UUGAUGAUU UGGGUUUU	471	AAAACCCA CUGAUGAG X CGAA AUCAUCAA	972
2887	UGAUGAUU GGGUUUUA	472	UAAAACCC CUGAUGAG X CGAA AAUCAUCA	973
2892	AUUUUGGGUU UUAUUUUU	473	AAAAUUA CUGAUGAG X CGAA ACCCAAAU	974
2893	UUUGGGGUU UAAUUUUG	474	AAAAAUUA CUGAUGAG X CGAA AACCCAAA	975
2894	UUGGGUUU AAUUUUGU	475	ACAAAAAU CUGAUGAG X CGAA AAACCCAA	976
2895	UGGGGUUUA AUUUUGUU	476	ACACAAA CUGAUGAG X CGAA AAAACCCA	977
2898	GUUUUUAUU UUGUUUUU	477	AAAAACAA CUGAUGAG X CGAA AUUAAAAC	978
2899	UUUUUAUUU UGUUUUUA	478	AAAAAAACA CUGAUGAG X CGAA AAUAAAAA	979
2900	UUUUAUUUU GUUUUUAU	479	AUAAAAAC CUGAUGAG X CGAA AAAUAAA	980
2903	AAUUUUGUU UUUAUUGC	480	GCAAUAAA CUGAUGAG X CGAA ACAAAAAU	981
2904	AUUUUGUUU UUAUUGCA	481	UGCAUAAA CUGAUGAG X CGAA AACAAAAU	982
2905	UUUUGUUUU UAUUGCAC	482	GUGCAUUA CUGAUGAG X CGAA AAACAAAA	983
2906	UUUGUUUUU AUUGCACC	483	GGUGCAAU CUGAUGAG X CGAA AAAACAAA	984
2907	UUGUUUUUA UUGCACCU	484	AGGUGCAA CUGAUGAG X CGAA AAAAACAA	985
2909	GUUUUCAUU GCACCUUGA	485	UCAGGUGC CUGAUGAG X CGAA AUAAAAC	986
2924	GACAAAAUA CAGUUUAUC	486	GAUAAUCG CUGAUGAG X CGAA AUUUUGUC	987
2929	AAUACAGUU AUCUGAUG	487	CAUCAGAU CUGAUGAG X CGAA ACUGUAU	988
2930	AUACAGUUU UCUGAUGG	488	CCAUCAGA CUGAUGAG X CGAA AACUGUAU	989
2932	ACAGUUUAUC UGAUGGUC	489	GACCAUCA CUGAUGAG X CGAA AUUACUGU	990
2940	CUGAUGGUC CCUCAAUU	490	AAUUGAGG CUGAUGAG X CGAA ACCAUUCAG	991
2944	UGGUCCCCUC AAAAAUGU	491	ACAUAAAU CUGAUGAG X CGAA AGGGACCA	992
2948	CCCUCAUUU AUGUUUAU	492	AAUAAACAU CUGAUGAG X CGAA AUUGAGGG	993
2949	CCUCAUUUA UGUUUAUUU	493	AAAUAACA CUGAUGAG X CGAA AAUUGAGG	994
2953	AAUUAUGUU AUUUUAAU	494	AUUAAAUAU CUGAUGAG X CGAA ACAUAAU	995
2954	AAUUAUGUUA UUUUAAUA	495	UAUUAAA CUGAUGAG X CGAA AACAUAAU	996
2956	UAUGUUAAU UUUAAA	496	UUUUAUUA CUGAUGAG X CGAA AUUACAU	997
2957	AUGUUUUU UAAUAAA	497	UUUUAUUA CUGAUGAG X CGAA AAUAACAU	998
2958	UGUUUUUUU AAUAAAUA	498	AUUUUAU CUGAUGAG X CGAA AAAUAACA	999
2959	GUUUUUUAU AAAAAUA	499	UAUUUUAU CUGAUGAG X CGAA AAAUAAC	1000
2962	AUUUUAAUA AAAUAAA	500	AUUUAAU CUGAUGAG X CGAA AUUAAA	1001
2967	AAUAAAUA AAUAAAUA	501	AUUUAAU CUGAUGAG X CGAA AUUUUAU	1002

Table XIII: Human C-ras Hairpin Ribozyme and Target Sequence

nt. Position	Ribozyme Sequence	SEQ ID. No.	Target Sequence	SEQ ID. No.
12	GCGGGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1003	UGACCCGCCUCCCGC	1078
19	UGAGGGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1004	CUCCCGCUCCCCUCA	1079
31	CCCCGGCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1005	CACCCGGCCGGGGC	1080
61	UUCGGCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1006	AAGCUCCGCCGCAA	1081
64	UCGGUCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1007	CUGCCGCCGAAACGA	1082
88	GAGCCAAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1008	GGGGGCGCCUGGUC	1083
205	UAGAUGCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1009	CUCCAUCGUCAUCU	1084
233	UAGCCAAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1010	CAGCAAUUGGCUAU	1085
258	GCCACUCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1011	CAUCAAUUAUUAU	1086
276	AGAAGGGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1012	UCACAUAUCCUUCU	1087
370	UCAUAAAAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1013	UGACUCCGUUAUGA	1088
427	GGGAGAAAGGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1014	GUUCAACUUCUC	1089
477	CGCAAGCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1015	AUACUCAUUGGCG	1090
605	CCAUUAGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1016	UUCUCCGUCAUUGG	1091
626	CCACAAAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1017	UGUCAACUUGGGG	1092
655	UGGGUGCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1018	GCACUUCGUACACCA	1093
789	CCUGGAGAGAAAGUACCUACCGAGAAACAXGUACAUUACCCGGUA	1019	AGUCUUCUCCAGG	1094
859	AUGAGGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1020	CUCUACGUCCUACU	1095
938	UCCACAAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1021	ACGGUCCGUUGGA	1096
990	AGGUUGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1022	AAUACUCCGUACACU	1097
1002	GGACAGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1023	CUUCACGUUCUCC	1098
1007	CUACUGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1024	GCCCCGUCCAGUAG	1099
1012	UGGGGCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1025	GUCCAUCGUACCCCA	1100
1049	GUUUUCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1026	UCACAUCCGCGAAC	1101
1089	CCCGAGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1027	CACCAUCGUACUGGG	1102
1181	CGAGUGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1028	AUGGUUCGUACUCG	1103
1190	GACCCAAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1029	ACUCGGAUUGGGUC	1104
1215	CUUAAAAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1030	GAACUUCGUUAUAGG	1105
1314	GCGCAAGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1031	UGGCCUUCGUUCGGCC	1106
1346	AUGAAAAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1032	AUUCUUCGUUUCAU	1107
1411	UGUAGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1033	CAGCAUCGUACUACA	1108
1451	UGGAACAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1034	UUUCAUCGUACUACA	1109
1481	UGAGGCCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1035	CGGGCAUCGUACUCA	1110
1485	UCCCCUGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1036	AGACGUCUAGGGAA	1111
1653	CCAGAGAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1037	GCUCUUCGUUCUGG	1112
1705	ACUGGAAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1038	AUUCUUCGUUCAGU	1113
1712	ACAUUCAGAAAGUACCGAGAAACAXGUACAUUACCCGGUA	1039	UUCCAUCGUUCAGU	1114

nt.	Position	Ribozyme Sequence	SEQ ID. No.	Target Sequence	SEQ ID. No.
1716	GUAGAC AGAA GACU ACCAGAGAAACA X GUACAUUACCUUGUA	1040	AGUCGG GAU GUCUAC	1115	
1751	CCCGUC AGAA GUUC ACCAGAGAAACA X GUACAUUACCUUGUA	1041	GAACU GAU GACGGG	1116	
1796	AAGAUG AGAA GAUC ACCAGAGAAACA X GUACAUUACCUUGUA	1042	GAUCA GAU CAUCU	1117	
1796	ACUAG AGAA GGGG ACCAGAGAAACA X GUACAUUACCUUGUA	1043	CCCCA GAU CUAJU	1118	
1833	ACUAG AGAA GUUC ACCAGAGAAACA X GUACAUUACCUUGUA	1044	GAACU GCC CCAAG	1119	
1858	CUUUGG AGAA GUUC ACCAGAGAAACA X GUACAUUACCUUGUA	1045	UAGCU GAC UGUUG	1120	
1887	CACACA AGAA GCUA ACCAGAGAAACA X GUACAUUACCUUGUA	1046	CCCCA GAU CCUGUC	1121	
1931	GACAGG AGAA GGGG ACCAGAGAAACA X GUACAUUACCUUGUA	1047	AUCCU GUC UCCAU	1122	
1937	AUGCAA AGAA GGAA ACCAGAGAAACA X GUACAUUACCUUGUA	1048	GAGCU GCU CCACACA	1123	
1952	UGUUGG AGAA GGUC ACCAGAGAAACA X GUACAUUACCUUGUA	1049	GGGCA GCC CACACU	1124	
2013	AGUCUG AGAA GCCC ACCAGAGAAACA X GUACAUUACCUUGUA	1050	ACGGCU GAC CACGUC	1125	
2045	GAACUG AGAA GCGU ACCAGAGAAACA X GUACAUUACCUUGUA	1051	AGGGCU GGC UGUU	1126	
2063	AAGACA AGAA GCCU ACCAGAGAAACA X GUACAUUACCUUGUA	1052	UGCCU GUC UUCUAG	1127	
2067	CUACAA AGAA GGCA ACCAGAGAAACA X GUACAUUACCUUGUA	1053	CACCU GUC UUCAGG	1128	
2090	CCUCAA AGAA GGUG ACCAGAGAAACA X GUACAUUACCUUGUA	1054	UUUCU GCU CCCU	1129	
2140	AAAGGG AGAA GAAA ACCAGAGAAACA X GUACAUUACCUUGUA	1055	AGACU GCU CACAGG	1130	
2204	CCUUGU AGAA GUUC ACCAGAGAAACA X GUACAUUACCUUGUA	1056	GUCCU GCU CCCUCC	1131	
2292	GGAGGG AGAA GGAC ACCAGAGAAACA X GUACAUUACCUUGUA	1057	GCCCCA GCC UUCUGU	1132	
2326	ACAGAA AGAA GGCG ACCAGAGAAACA X GUACAUUACCUUGUA	1058	CUUCU GUA GAUGGG	1133	
2333	CGCAUC AGAA GAAG ACCAGAGAAACA X GUACAUUACCUUGUA	1059	GCCCCA GCC CCAGCU	1134	
2381	AGCUGG AGAA GGCG ACCAGAGAAACA X GUACAUUACCUUGUA	1060	CCCCA GCU GUUGGC	1135	
2387	GCCAAC AGAA GGGG ACCAGAGAAACA X GUACAUUACCUUGUA	1061	CAGCU GCU GGCUAC	1136	
2390	GUAGCC AGAA GCUG ACCAGAGAAACA X GUACAUUACCUUGUA	1062	AGGCA GUC CAGCCC	1137	
2431	GGGCUG AGAA GCCU ACCAGAGAAACA X GUACAUUACCUUGUA	1063	GUCCA GCC CUGAUG	1138	
2436	CAUCAG AGAA GGAC ACCAGAGAAACA X GUACAUUACCUUGUA	1064	GGCCC GCC CGAAGG	1139	
2441	CUCAC AGAA GGGC ACCAGAGAAACA X GUACAUUACCUUGUA	1065	AAUCA GCU UCGGGA	1140	
2472	UCCAGA AGAA GAUU ACCAGAGAAACA X GUACAUUACCUUGUA	1066	AAACA GCC CAGGAC	1141	
2557	GUUCUG AGAA GUUU ACCAGAGAAACA X GUACAUUACCUUGUA	1067	GGACU GCC GAGACU	1142	
2567	AGUCUC AGAA GUCC ACCAGAGAAACA X GUACAUUACCUUGUA	1068	UGGCC GCC CGAAGG	1143	
2582	CCUUCG AGAA GCCA ACCAGAGAAACA X GUACAUUACCUUGUA	1069	AGCCU GCU UGGUA	1144	
2596	UACCAA AGAA GGCU ACCAGAGAAACA X GUACAUUACCUUGUA	1070	UCRCA GCU UCUAAG	1145	
2644	CUUAGA AGAA GUUG ACCAGAGAAACA X GUACAUUACCUUGUA	1071	UCUCA GCC CUCU	1146	
2710	UGAGAG AGAA GAGA ACCAGAGAAACA X GUACAUUACCUUGUA	1072	GAGCA GUC UCCAU	1147	
2725	AUGGAA AGAA GCUC ACCAGAGAAACA X GUACAUUACCUUGUA	1073	GAGCU GCC CCU	1148	
2761	CAUAGG AGAA GGUC ACCAGAGAAACA X GUACAUUACCUUGUA	1074	GGCCA GCC UGUUC	1149	
2788	GAACA AGAA GGCC ACCAGAGAAACA X GUACAUUACCUUGUA	1075	AGCCU GUU UCUCUA	1150	
2792	UAGAGA AGAA GGGU ACCAGAGAAACA X GUACAUUACCUUGUA	1076	AAACCA GCC UGGUU	1151	
2820	AAACAA AGAA GUUU ACCAGAGAAACA X GUACAUUACCUUGUA	1077	UAUCU GAU GGUCC	1152	
2933	GGGACC AGAA GAUA ACCAGAGAAACA X GUACAUUACCUUGUA				

Where "X" represents stem IV region of a Hairpin ribozyme. The length of stem IV may be 2 base-pairs.

Table XIV. Hammerhead Rib zyme Sites for A-Raf

Pos	RZ	SEQ ID. No.	Substrate	SEQ ID. No.
10	UCCACCCU CUGAUGAG X CGAA AUUGGGUC	1153	GACCCAAUA AGGGUGGA	1461
28	CUCUGCGG CUGAUGAG X CGAA ACUCAGCC	1154	GGCUGAGUC CCGCAGAG	1462
42	ACUCUCGU CUGAUGAG X CGAA AUUGGCUC	1155	GAGCCAAUA ACGAGAGU	1463
51	GCCUCUCG CUGAUGAG X CGAA ACUCUCGU	1156	ACGAGAGUC CGAGAGGC	1464
74	UCCUCACA CUGAUGAG X CGAA AGUCCGCC	1157	GGCGGACUC UGUGAGGA	1465
123	CACGCCGC CUGAUGAG X CGAA ACAGCCGC	1158	GCGGCGUUA GCGCGUG	1466
166	GAUGGGCU CUGAUGAG X CGAA AGGUGGGG	1159	CCCCACCUA AGCCAUC	1467
174	UUUGUCAA CUGAUGAG X CGAA AUGGGCUG	1160	CAGCCCAUC UUGACAAA	1468
176	AUUUUGUC CUGAUGAG X CGAA AGAUGGGC	1161	GCCCAUCUU GACAAAAU	1469
185	GAGCCUUA CUGAUGAG X CGAA AUUUUGUC	1162	GACAAAUC UAAGGCUC	1470
187	UGGAGCCU CUGAUGAG X CGAA AGAUUUUG	1163	CAAAAUCUA AGGCUCCA	1471
193	GCUCCAUG CUGAUGAG X CGAA AGCCUUAG	1164	CUAAGGCUC CAUGGAGC	1472
238	CUGCCCGG CUGAUGAG X CGAA AUGGCUCG	1165	CGAGCCAUC CCGGGCAG	1473
257	UAUACUUU CUGAUGAG X CGAA ACGGUGCC	1166	GGCACCGUC AAAGUAUA	1474
263	GGCAGGUA CUGAUGAG X CGAA ACUUUGAC	1167	GUCAAAGUA UACCUGCC	1475
265	UGGGCAGG CUGAUGAG X CGAA AUACUUUG	1168	CAAAGUUA CCUGCCCA	1476
299	CCAUCCCG CUGAUGAG X CGAA ACAGUCAC	1169	GUGACUGUC CGGGAUGG	1477
317	GAGUCGUA CUGAUGAG X CGAA ACACUCAU	1170	AUGAGUGUC UACCGACUC	1478
319	GAGAGUCG CUGAUGAG X CGAA AGACACUC	1171	GAGUGUCUA CGACUCUC	1479
325	UGUCUAGA CUGAUGAG X CGAA AGUCGUAG	1172	CUACGACUC UCUAGACA	1480
327	CUUUGUCUA CUGAUGAG X CGAA AGAGUCGU	1173	ACGACUCUC UAGACAAG	1481
329	GCCUUGUC CUGAUGAG X CGAA AGAGAGUC	1174	GACUCUCUA GACAAGGC	1482
354	CUGAUUUU CUGAUGAG X CGAA ACCCCGCA	1175	UGCGGGGUC UAAAUCAG	1483
356	UCCUGAUU CUGAUGAG X CGAA AGACCCCG	1176	CGGGGUCUA AAUCAGGA	1484
360	GCAGUCCU CUGAUGAG X CGAA AUUUAGAC	1177	GUCUAAAUC AGGACUGC	1485
377	AGUCGGUA CUGAUGAG X CGAA ACCACACA	1178	UGUGUGGUC UACCGACU	1486
379	UGAGUCGG CUGAUGAG X CGAA AGACACACA	1179	UGUGGUCUA CCGACCUA	1487
386	CCCUUGAU CUGAUGAG X CGAA AGUCGGUA	1180	UACCGACUC AUCAAGGG	1488
389	CGUCCCCU CUGAUGAG X CGAA AUGAGUCG	1181	CGACUCUAUC AAGGGACG	1489
407	CAGGCAGU CUGAUGAG X CGAA ACCGUCUU	1182	AAGACGGUC ACUGCCUG	1490
428	AGGGGAGC CUGAUGAG X CGAA AUGGCUGU	1183	ACAGCCAUU GCUCCCU	1491
432	AUCCAGGG CUGAUGAG X CGAA AGCAAUUGG	1184	CCAUUGGCUC CCCUGGAU	1492
452	UCGACAAU CUGAUGAG X CGAA AGCUCCUC	1185	GAGGAGGCUC AUUGUCGA	1493
455	ACCUUCGAC CUGAUGAG X CGAA AUGAGCUC	1186	GAGCUCAUU GUCGAGGU	1494
458	AGGACCUUC CUGAUGAG X CGAA ACAAUUGAG	1187	CUCAUUGUC GAGGUCCU	1495
464	UCUUCAAG CUGAUGAG X CGAA ACCUCGAC	1188	GUCGAGGUC CUUGAAGA	1496
467	ACAUCUUC CUGAUGAG X CGAA AGGACUC	1189	GAGGUCCUU GAAGAUGU	1497
476	GUCAGCGG CUGAUGAG X CGAA ACAUCUUC	1190	GAAGAUGUC CCGCUGAC	1498
495	CCGUACAA CUGAUGAG X CGAA AUUGUGCA	1191	UGCACAAUU UUGUACGG	1499
496	UCCGUACA CUGAUGAG X CGAA AAUUGUGC	1192	GCACAAUUU UGUACGGA	1500
497	UCCGUAC CUGAUGAG X CGAA AAAUUGUG	1193	CACAAUUU GUACGGAA	1501
500	GUCUUCGG CUGAUGAG X CGAA ACAAAAUU	1194	AAUUUUGUA CGGAAGAC	1502
511	GGCUGAAG CUGAUGAG X CGAA AGGUCCU	1195	GAAGACCUU CUUCAGCC	1503
512	AGGCUGAA CUGAUGAG X CGAA AAGGUCCU	1196	AAGACCUUC UUCAGCCU	1504
514	CCAGGCUG CUGAUGAG X CGAA AGAAGGUC	1197	GACCUUCUU CAGCCUGG	1505
515	GCCAGGCCU CUGAUGAG X CGAA AAGAAGGU	1198	ACCUUCUUC AGCCUGGC	1506
526	AGUCACAG CUGAUGAG X CGAA ACGCCAGG	1199	CCUGGCGUU CUGUGACU	1507
527	AAGUCACA CUGAUGAG X CGAA AACGCCAG	1200	CUGGCGUUC UGUGACUU	1508
535	UAAGGCAG CUGAUGAG X CGAA AGUCACAG	1201	CUGUGACUU CUGCCUUA	1509
536	UUAAGGCA CUGAUGAG X CGAA AAGUCACA	1202	UGUGACUUC UGCCUUAA	1510
542	AGAAACUU CUGAUGAG X CGAA AGGCAGAA	1203	UUCUGCCUU AAGUUUCU	1511
543	CAGAAACU CUGAUGAG X CGAA AAGGCAGA	1204	UCUGCCUUA AGUUUCUG	1512

Pos	RZ	SEQ ID. No.	Substrate	SEQ ID. No.
547	GGAACAGA CUGAUGAG X CGAA ACUUAAGG	1205	CCUUAAGUU UCUGUUCC	1513
548	UGGAACAG CUGAUGAG X CGAA AACUUAAG	1206	CUUAAGUUU CUGUUC	1514
549	AUGGAACA CUGAUGAG X CGAA AAACUUA	1207	UUAAGUUUC UGUUCCAU	1515
553	AGCAUAGG CUGAUGAG X CGAA ACAGAAC	1208	GUUUCUGUU CCAUCCU	1516
554	AAGCCAUG CUGAUGAG X CGAA AACAGAAA	1209	UUUCUGUUC CAUGGCUU	1517
562	GGCAACGG CUGAUGAG X CGAA AGCCAUGG	1210	CCAUGGCUU CCGUUGCC	1518
563	UGGCAACG CUGAUGAG X CGAA AAGCCAUG	1211	CAUGGCUUC CGUUGCCA	1519
567	GGUUGGC CUGAUGAG X CGAA ACGGAAGC	1212	GCUUCCGUU GCCAAACC	1520
583	GGAACUUG CUGAUGAG X CGAA AGCCACAG	1213	CUGUGGCUA CAAGUCC	1521
589	GCUGGUGG CUGAUGAG X CGAA ACUUGUAG	1214	CUACAAGUU CCACCAGC	1522
590	UGCUGGUG CUGAUGAG X CGAA AACUUGUA	1215	UACAAGUUC CACCAGCA	1523
600	GGAGGAAC CUGAUGAG X CGAA AUGCUGGU	1216	ACCAGCAUU GUUCCUCC	1524
603	CUUGGAGG CUGAUGAG X CGAA ACAAUGC	1217	AGCAUUGUU CCUCCAAG	1525
604	CCUUGGAG CUGAUGAG X CGAA AACAAUGC	1218	GCAUUGUUC CUCCAAGG	1526
607	GGACCUUG CUGAUGAG X CGAA AGGAACAA	1219	UUGUUCCUC CAAGGUCC	1527
614	AC JUGGG CUGAUGAG X CGAA ACCUUGGA	1220	UCCAAGGUC CCCACAGU	1528
623	UCACACACA CUGAUGAG X CGAA ACUGUGGG	1221	CCCACAGUC UGUGUUGA	1529
629	CUCAUGUC CUGAUGAG X CGAA ACACAGAC	1222	GUCUGUUU GACAUGAG	1530
639	GCGGUUGG CUGAUGAG X CGAA ACUCAUGU	1223	ACAUGAGUA CCAACCGC	1531
655	UGUGGUAG CUGAUGAG X CGAA ACUGUUGG	1224	CCAACAGUU CUACCAACA	1532
656	CUGUGGUA CUGAUGAG X CGAA AACUGUUG	1225	CAACAGUUC UACCACAG	1533
658	CACUGUGG CUGAUGAG X CGAA AGAACUGU	1226	ACAGUUCUA CCACAGUG	1534
668	AAAUCUCG CUGAUGAG X CGAA ACACUGUG	1227	CACAGUGUC CAGGAUUU	1535
675	UCCGGACA CUGAUGAG X CGAA AUCCUGGA	1228	UCCAGGAUU UGUCCGGA	1536
676	CUCCGGAC CUGAUGAG X CGAA AAUCCUGG	1229	CCAGGAUUU GUCCGGAG	1537
679	AGCCUCCG CUGAUGAG X CGAA ACAAAUC	1230	GGAUUUGUC CGGAGGCU	1538
688	GCUGUCUG CUGAUGAG X CGAA AGCCUCGG	1231	CGGAGGCUC CAGACAGC	1539
705	GUUCGGAGG CUGAUGAG X CGAA AGCCUCAU	1232	AUGAGGCUC CCUCGAAC	1540
709	GGCGGUUC CUGAUGAG X CGAA AGGGAGCC	1233	GGCUCCUC GAACCGCC	1541
730	GGGUUAGC CUGAUGAG X CGAA ACUCAUUC	1234	GAAUGAGUU GCUAACCC	1542
734	UGGGGGGU CUGAUGAG X CGAA AGCAACUC	1235	GAGUUGCUA ACCCCCCA	1543
747	GGGGCUGG CUGAUGAG X CGAA ACCCUGGG	1236	CCCAGGGUC CCAGCCCC	1544
784	GGAAGGGG CUGAUGAG X CGAA AGUGCUC	1237	GGAGCACUU CCCUUC	1545
785	GGGAAGGG CUGAUGAG X CGAA AAGUGCUC	1238	GAGCACUUC CCCUUC	1546
790	GGGCAGGG CUGAUGAG X CGAA AGGGGAAG	1239	CUUCCCCUU CCCUGCCC	1547
791	GGGGCAGG CUGAUGAG X CGAA AAGGGGAA	1240	UUCCCCUUC CCUGCCCC	1548
815	AUGCGCUG CUGAUGAG X CGAA AGGGGGGC	1241	GCCCCCCUA CAGCGCAU	1549
824	GUGGAGCG CUGAUGAG X CGAA AUGCGCUG	1242	CAGCGCAUC CGCUCCAC	1550
829	UGGACGUG CUGAUGAG X CGAA AGCGGAUG	1243	CAUCCGCUC CACGUCCA	1551
835	UGGGAGUG CUGAUGAG X CGAA ACGUGGAG	1244	CUCCACGUC CACUCCCA	1552
840	GACGUUGG CUGAUGAG X CGAA AGUGGACG	1245	CGUCCACUC CCAACGUC	1553
848	ACCAUAUG CUGAUGAG X CGAA ACGUUGGG	1246	CCCAACGUC CAUAUGGU	1554
852	GCUGACCA CUGAUGAG X CGAA AUGGACGU	1247	ACGUCCAUA UGGUCAGC	1555
857	GUGGUGCU CUGAUGAG X CGAA ACCAUAG	1248	CAUAUGGUC AGCACCAC	1556
880	UGAGGUUG CUGAUGAG X CGAA AGUCCAU	1249	CAUGGACUC CAACCUA	1557
887	AGCUGGAU CUGAUGAG X CGAA AGGUUGGA	1250	UCCAACCUC AUCCAGCU	1558
890	GUGAGCUG CUGAUGAG X CGAA AUGAGGU	1251	AACCUCAU CAGCUCAC	1559
896	UGGCCAGU CUGAUGAG X CGAA AGCUGGAU	1252	AUCCAGCUC ACUGGCCA	1560
909	AGUGCUGA CUGAUGAG X CGAA ACUCUGGC	1253	GCCAGAGUU UCAGCACU	1561
910	CAGUGCUG CUGAUGAG X CGAA AACUCUGG	1254	CCAGAGUUU CAGCACUG	1562
911	UCAGUGCU CUGAUGAG X CGAA AAACUCUG	1255	CAGAGUUUC AGCACUGA	1563
930	UCCUCUAC CUGAUGAG X CGAA ACCGGCAG	1256	CUGCCGGUA GUAGAGGA	1564
933	ACCUCCUC CUGAUGAG X CGAA ACUACCGG	1257	CCGGUAGUA GAGGAGGU	1565
942	UCCAUCAC CUGAUGAG X CGAA ACCUCCUC	1258	GAGGAGGU GUGAUGGA	1566

Pos	RZ	SEQ ID. No.	Substrate	SEQ ID. No.
985	UCCCCGAG CUGAUGAG X CGAA ACACGCUG	1259	CAGCGUGUC CUCGGGGA	1567
988	UCCUCCCC CUGAUGAG X CGAA AGGACACG	1260	CGUGUCCUC GGGGAGGA	1568
1000	AAUGUGGG CUGAUGAG X CGAA ACUUCCUC	1261	GAGGAAGUC CCCACAUU	1569
1008	UGACUUGG CUGAUGAG X CGAA AUGUGGGG	1262	CCCCACAUU CCAAGUCA	1570
1009	GUGACUUG CUGAUGAG X CGAA AAUGUGGG	1263	CCCACAUUC CAAGUCAC	1571
1015	CUGCUGGU CUGAUGAG X CGAA ACUUGGAA	1264	UUCCAAGUC ACCAGCAG	1572
1042	CGGCCAAG CUGAUGAG X CGAA ACUUCCGC	1265	GCGGAAGUC CUUGGCCG	1573
1045	CAUCGGCC CUGAUGAG X CGAA AGGACUUC	1266	GAAGUCCUU GGCGAUG	1574
1081	ANUCCCCG CUGAUGAG X CGAA ACCCCAGG	1267	CCUGGGGUA CCGGGANU	1575
1090	AAUAGCCU CUGAUGAG X CGAA ANUCCCCG	1268	CCGGGANUC AGGCUAUU	1576
1096	CCCAGUAA CUGAUGAG X CGAA AGCCUGAN	1269	NUCAGGCUA UUACUGGG	1577
1098	CUCCCAGU CUGAUGAG X CGAA AUAGCCUG	1270	CAGGCUAUU ACUGGGAG	1578
1099	CCUCCCGAG CUGAUGAG X CGAA AAUAGCCU	1271	AGGCUAUUA CUGGGAGG	1579
1109	CUGGGUGG CUGAUGAG X CGAA ACCUCCA	1272	UGGGAGGUA CCACCCAG	1580
1142	CCCGUCCC CUGAUGAG X CGAA AUCCUCUU	1273	AAGAGGAUC GGGACGGG	1581
1153	UGCCAAAC CUGAUGAG X CGAA AGCCCGUC	1274	GACGGGCUC GUUUGGCA	1582
1156	CGGUGCCA CUGAUGAG X CGAA ACGAGCCC	1275	GGGCUCGUU UGGCACCG	1583
1157	ACGGUGCC CUGAUGAG X CGAA AACGAGCC	1276	GGCUCGUUU GGCACCGU	1584
1168	GCCUCUGA CUGAUGAG X CGAA ACACGGUG	1277	CACCGUGUU UCGAGGGC	1585
1169	CGCCCUCG CUGAUGAG X CGAA AACACGGU	1278	ACCGUGUUU CGAGGGCG	1586
1170	CGGCCCCU CUGAUGAG X CGAA AAACACGG	1279	CCGUGUUUC GAGGGCGG	1587
1208	GACACCUU CUGAUGAG X CGAA AGCACCUU	1280	AAGGUGCUC AAGGUGUC	1588
1216	UGGGCUGG CUGAUGAG X CGAA ACACCUUG	1281	CAAGGUGUC CCAGCCCA	1589
1245	AUUCUUGA CUGAUGAG X CGAA AGCCUGGG	1282	CCCAGGCUU UCAAGAAU	1590
1246	CAUUCUUG CUGAUGAG X CGAA AAGCCUGG	1283	CCAGGCUUU CAAGAAUG	1591
1247	UCAUUCUU CUGAUGAG X CGAA AAAGCCUG	1284	CAGGCUUUC AAGAAUGA	1592
1268	GUCUUCCU CUGAUGAG X CGAA AGCACCUG	1285	CAGGUGCUC AGGAAGAC	1593
1286	AAGAUGUU CUGAUGAG X CGAA ACAUGUCC	1286	CGACAUUC AACAUUU	1594
1292	AACAGCAA CUGAUGAG X CGAA AUGUUGAC	1287	GUCAACAU UUGCUGUU	1595
1294	UAAACAGC CUGAUGAG X CGAA AGAUGUUG	1288	CAACAUUU GCUGUUUA	1596
1300	AGCCCCAU CUGAUGAG X CGAA ACAGCAAG	1289	CUUGCUGUU UAUGGGCU	1597
1301	AAGCCCCA CUGAUGAG X CGAA AACAGCAA	1290	UUGCUGUUU AUGGGCUU	1598
1302	GAAGCCCA CUGAUGAG X CGAA AAACAGCA	1291	UGCUGUUUA UGGGCUUC	1599
1309	GGGUCAUG CUGAUGAG X CGAA AGCCCCAU	1292	UAUGGGCUU CAUGACCC	1600
1310	CGGGUCAU CUGAUGAG X CGAA AAGCCCCAU	1293	AUGGGCUUC AUGACCCG	1601
1327	UGAUGGCA CUGAUGAG X CGAA AUCCCCGG	1294	GCCGGGAUU UGCCAUCA	1602
1328	AUGAUGGC CUGAUGAG X CGAA AAUCCCCG	1295	CCGGGAAUUU GCCAUCAU	1603
1334	UGUGUGAU CUGAUGAG X CGAA AUGGCAAA	1296	UUUGCCAUC AUCACACA	1604
1337	CACUGUGU CUGAUGAG X CGAA AUGAUGGC	1297	GCCAUCAU ACACAGUG	1605
1357	AGAGGCUG CUGAUGAG X CGAA AGCCCUCA	1298	UGAGGGCUC CAGCCUCU	1606
1364	UGAUGGUA CUGAUGAG X CGAA AGGCUGGA	1299	UCCAGCCUC UACCAUCA	1607
1366	GGUGAUGG CUGAUGAG X CGAA AGAGGCUG	1300	CAGCCUCUA CCAUCACC	1608
1371	AUGCAGGU CUGAUGAG X CGAA AUGGUAGA	1301	UCUACCAUC ACCUGCAU	1609
1396	CCAUGUCG CUGAUGAG X CGAA AGCGUGUG	1302	CACACGCUU CGACAUGG	1610
1397	ACCAUGUC CUGAUGAG X CGAA AAGCGUGU	1303	ACACGCUUC GACAUGGU	1611
1406	AUGAGCUG CUGAUGAG X CGAA ACCAUGUC	1304	GACAUGGUC CAGCUCAU	1612
1412	ACGUCGAU CUGAUGAG X CGAA AGCUGGAC	1305	GUCCAGCUC AUCCACGU	1613
1415	GCCACGUC CUGAUGAG X CGAA AUGAGCUG	1306	CAGCUACU CAGGUGGC	1614
1450	CAUGGAGG CUGAUGAG X CGAA AGUCCAU	1307	CAUGGACUA CCUCCAUG	1615
1454	UUGGCAUG CUGAUGAG X CGAA AGGUAGUC	1308	GACUACCU C AUGCCAA	1616
1469	CGGUGGAU CUGAUGAG X CGAA AUGUUCUU	1309	AAGAACAUU AUCCACCG	1617
1472	UCUCGGUG CUGAUGAG X CGAA AUGAUGUU	1310	AAACAUCAUC CACCGAGA	1618
1482	AGACUUGA CUGAUGAG X CGAA AUCUCGGU	1311	ACCGAGAUC UCAAGUCU	1619
1484	UUAGACUU CUGAUGAG X CGAA AGAUCUCG	1312	CGAGAUCUC AAGUCUAA	1620

Pos	RZ	SEQ ID. No.	Substrate	SEQ ID. No.
1489	UGUUGUUA CUGAUGAG X CGAA ACUUGAGA	1313	UCUCAAGUC UAACAACA	1621
1491	GAUGUUGU CUGAUGAG X CGAA AGACUUGA	1314	UCAAGUCUA ACAACAUC	1622
1499	UGUAGGAA CUGAUGAG X CGAA AUGUUGUU	1315	AAACAACAU UUCCUACA	1623
1501	CAUGUAGG CUGAUGAG X CGAA AGAUGUUG	1316	AAACAUCUU CCUACAUG	1624
1502	UCAUGUAG CUGAUGAG X CGAA AAGAUGUU	1317	AAACAUCUUC CUACAUGA	1625
1505	CCCUCAUG CUGAUGAG X CGAA AGGAAGAU	1318	AUCUUCCUA CAUGAGGG	1626
1517	UUCACCGU CUGAUGAG X CGAA AGCCCCUC	1319	GAGGGCUC ACGGUGAA	1627
1529	AAGUCACC CUGAUGAG X CGAA AUCUUCAC	1320	GUGAAGAUC GGUGACUU	1628
1537	CCAAGCCA CUGAUGAG X CGAA AGUCACCG	1321	CGGUGACUU UGGCUUGG	1629
1538	GCCAAGCC CUGAUGAG X CGAA AAGUCACC	1322	GGUGACUUU GGCUUGGC	1630
1543	CUGUGGCC CUGAUGAG X CGAA AGCCAAAG	1323	CUUUGGCUU GGCCACAG	1631
1560	GCUCCAUC CUGAUGAG X CGAA AGUCUUCA	1324	UGAAGACUC GAUGGAGC	1632
1582	GCUGCUCC CUGAUGAG X CGAA AGGGCUGG	1325	CCAGCCCUU GGAGCAGC	1633
1594	CAGAUCCU CUGAUGAG X CGAA AGGGCUGC	1326	GCAGCCCUC AGGAUCUG	1634
1600	ACAGCACA CUGAUGAG X CGAA AUCCUGAG	1327	CUCAGGAUC UGUGCUGU	1635
1628	UGCAUACG CUGAUGAG X CGAA AUCACCUC	1328	GAGGUGAUC CGUAUGCA	1636
1632	GUCCUGCA CUGAUGAG X CGAA ACGGAUCA	1329	UGAUCCGUA UGCAGGAC	1637
1651	GGAAGCUG CUGAUGAG X CGAA AGGGGUUC	1330	GAACCCUA CAGCUUCC	1638
1657	CUGACUGG CUGAUGAG X CGAA AGCUGUAG	1331	CUACAGCUU CCAGUCAG	1639
1658	UCUGACUG CUGAUGAG X CGAA AAGCUGUA	1332	UACAGCUUC CAGUCAGA	1640
1663	AGACGUCU CUGAUGAG X CGAA ACUGGAAG	1333	CUUCCAGUC AGACGUCU	1641
1670	UAGGCAUA CUGAUGAG X CGAA ACGUCUGA	1334	UCAGACGUC UAUGCCUA	1642
1672	CGUAGGCA CUGAUGAG X CGAA AGACGUCU	1335	AGACGUCUA UGCCUACG	1643
1678	CAACCCCG CUGAUGAG X CGAA AGGCAUAG	1336	CUAUGCCUA CGGGGUUG	1644
1685	UAGAGCAC CUGAUGAG X CGAA ACCCCGUA	1337	UACGGGUU GUGCUCUA	1645
1691	AGCUCGUA CUGAUGAG X CGAA AGCACAAAC	1338	GUUGUGCUC UACGAGCU	1646
1693	UAAGCUCG CUGAUGAG X CGAA AGAGCACCA	1339	UGUGCUCUA CGAGCUUA	1647
1700	CCAGUCAU CUGAUGAG X CGAA AGCUCGUA	1340	UACGAGCUU AUGACUGG	1648
1701	GCCAGUCA CUGAUGAG X CGAA AAGCUCGU	1341	ACGAGCUUA UGACUGGC	1649
1711	AAGGCAGU CUGAUGAG X CGAA AGCCAGUC	1342	GACUGGCUC ACUGCCUU	1650
1719	GUGGCUGU CUGAUGAG X CGAA AGGCAGUG	1343	CACUGCCUU ACAGCCAC	1651
1720	UGUGGCUG CUGAUGAG X CGAA AAGGCAGU	1344	ACUGCCUU AAGCCACA	1652
1730	CGGCAGCC CUGAUGAG X CGAA AUGUGGCU	1345	AGCCACAUU GGCUGCCG	1653
1748	AUAAAAGAU CUGAUGAG X CGAA AUCUGGUC	1346	GACCAGAUU AUCUUUAU	1654
1749	CAUAAAAGA CUGAUGAG X CGAA AAUCUGGU	1347	ACCAGAUUA UCUUUUAUG	1655
1751	ACCAUAAA CUGAUGAG X CGAA AUAAAUCUG	1348	CAGAUUAUC UUUUUGGU	1656
1753	CCACCAUA CUGAUGAG X CGAA AGAUAAAUC	1349	GAUUAUCUU UAUGGUGG	1657
1754	CCCACCAU CUGAUGAG X CGAA AAGAUAAU	1350	AUUAUCUUU AUGGUGGG	1658
1755	GCCCCACCA CUGAUGAG X CGAA AAAGAUAA	1351	UUUAUCUUUA UGGUGGGC	1659
1771	GGGACAGA CUGAUGAG X CGAA AGCCACGG	1352	CCGUGGCUA UCUGUCCC	1660
1773	CGGGGACA CUGAUGAG X CGAA AUAGCCAC	1353	GUGGCUAUC UGUCCCCG	1661
1777	GGUCCGGG CUGAUGAG X CGAA ACAGAUAG	1354	CUAUCUGUC CCCGGACC	1662
1787	AUUUUGCU CUGAUGAG X CGAA AGGUCCGG	1355	CCGGACCUC AGCAAAAU	1663
1796	UUGCUGGA CUGAUGAG X CGAA AUUUUGCU	1356	AGCAAAUUC UCCAGCAA	1664
1798	AGUUGCUG CUGAUGAG X CGAA AGAuuuUG	1357	CAAAAUUC CAGCAACU	1665
1834	GGCAGUCA CUGAUGAG X CGAA ACAGCAGG	1358	CCUGCUGUC UGACUGCC	1666
1844	UGGAACUU CUGAUGAG X CGAA AGGCAGUC	1359	GACUGCCUC AAGUUCCA	1667
1849	CCCGCUGG CUGAUGAG X CGAA ACUUGAGG	1360	CCUCAAGUU CCAGCGGG	1668
1850	UCCCGCUG CUGAUGAG X CGAA AACUUGAG	1361	CUCAAGUUC CAGCGGGGA	1669
1871	UGGGGGAA CUGAUGAG X CGAA AGGGGCCG	1362	CGGCCCCUC UUCCCCCA	1670
1873	UCUGGGGG CUGAUGAG X CGAA AGAGGGC	1363	GCCCCUCUU CCCCCAGA	1671
1874	AUCUGGGG CUGAUGAG X CGAA AAGAGGGG	1364	CCCCUCUUC CCCCCAGAU	1672
1883	GUGGCCAG CUGAUGAG X CGAA AUCUGGGG	1365	CCCCAGAUC CUGGCCAC	1673
1895	AGCAGCUC CUGAUGAG X CGAA AUUGUGGC	1366	GCCACAAUU GAGCUGCU	1674

Pos	RZ	SEQ ID. No.	Substrat	SEQ ID. No.
1912	UGGGGAGU CUGAUGAG X CGAA ACCGUUGC	1367	GCAACGGUC ACUCCCCA	1675
1916	AUCUUGGG CUGAUGAG X CGAA AGUGACCG	1368	CGGUCACUC CCCAAGAU	1676
1925	CUCCGCUC CUGAUGAG X CGAA AUCUUGGG	1369	CCCAAGAUU GAGCGGAG	1677
1939	AGGGUUCC CUGAUGAG X CGAA AGGCACUC	1370	GAGUGCCUC GGAACCCU	1678
1948	GGUGCAAG CUGAUGAG X CGAA AGGGJUCC	1371	GGAACCCUC CUUGCACC	1679
1951	UGCAGGUC CUGAUGAG X CGAA AGGAGGGU	1372	ACCCUCCU GCACCGCA	1680
1975	AGGCAGGC CUGAUGAG X CGAA ACUCAUCG	1373	CUGAUGAGUU GCCUGCCU	1681
1988	GCGCUGAG CUGAUGAG X CGAA AGGCAGGC	1374	GCCUGCCUA CUCAGCGC	1682
1991	GCUGCGCU CUGAUGAG X CGAA AGUAGGGC	1375	UGCCUACUC AGGCAGC	1683
2006	UAAGGCAC CUGAUGAG X CGAA AGGCGGGC	1376	GCCCCGCCU GUGCCUUA	1684
2013	CGGGGCCU CUGAUGAG X CGAA AGGCACAA	1377	UUGUGCCU AGGCCCCG	1685
2014	GCGGGGCC CUGAUGAG X CGAA AAGGCACA	1378	UGUGCCUUA GGCCCCGC	1686
2044	AGGGCUGA CUGAUGAG X CGAA AUUGGCUC	1379	GAGCCAAUC UCAGCCCU	1687
2046	GGAGGGCU CUGAUGAG X CGAA AGAUUGGC	1380	GCCAAUCUC AGCCCUCC	1688
2053	UUGGCGUG CUGAUGAG X CGAA AGGGCUGA	1381	UCAGCCUC CACGCCAA	1689
2069	UGGUGGGC CUGAUGAG X CGAA AGGCUCCU	1382	AGGAGCCU GCCCACCA	1690
2084	CGAACAUU CUGAUGAG X CGAA AUUGGCUG	1383	CAGCCAAUC AAUGUUCG	1691
2090	CAGAGACG CUGAUGAG X CGAA ACAUUGAU	1384	AUCAAUGUU CGUCUCUG	1692
2091	GCAGAGAC CUGAUGAG X CGAA AACAUUGA	1385	UCAAUGUUC GUCUCUGC	1693
2094	AGGGCAGA CUGAUGAG X CGAA ACGAACAU	1386	AUGUUCGUC UCUGCCU	1694
2096	UCAGGGCA CUGAUGAG X CGAA AGACGAAC	1387	GUUCGUCUC UGCCCCUGA	1695
2113	GGGAUCCU CUGAUGAG X CGAA AGGCAGCA	1388	UGCUGCCUC AGGAUCCC	1696
2119	GAAUGGGG CUGAUGAG X CGAA AUCCUGAG	1389	CUCAGGAUC CCCCAUUC	1697
2126	GGGUGGGG CUGAUGAG X CGAA AUGGGGGA	1390	UCCCCCAUU CCCCACCC	1698
2127	AGGGUGGG CUGAUGAG X CGAA AAUGGGGG	1391	CCCCCAUUC CCCACCCU	1699
2151	CACAUGGG CUGAUGAG X CGAA ACCCCCCUC	1392	GAGGGGGUC CCCAUGUG	1700
2162	AACUGGAA CUGAUGAG X CGAA AGCACAUG	1393	CAUGUGCUU UCCAGUU	1701
2163	GAACUGGA CUGAUGAG X CGAA AAGCACAU	1394	AUGUGCUUU UCCAGUUC	1702
2164	AGAACUGG CUGAUGAG X CGAA AAAGCACA	1395	UGUGCUUUU CCAGUUCU	1703
2165	AAGAACUG CUGAUGAG X CGAA AAAAGCAC	1396	GUGCUUUUC CAGUUCUU	1704
2170	UCCAGAAG CUGAUGAG X CGAA ACUGGAAA	1397	UUUCCAGUU CUUCUGGA	1705
2171	UUCCAGAA CUGAUGAG X CGAA AACUGGAA	1398	UUCCAGUUC UUCUGGAA	1706
2173	AAUUCCAG CUGAUGAG X CGAA AGAACUGG	1399	CCAGUUCUU CUGGAAUU	1707
2174	CAAUUCCA CUGAUGAG X CGAA AAGAACUG	1400	CAGUUCUUC UGGAAUUG	1708
2181	GGUCCCCC CUGAUGAG X CGAA AUUCCAGA	1401	UCUGGAAUU GGGGGACC	1709
2214	AUGGAGGA CUGAUGAG X CGAA ACAGGGGG	1402	CCCCCUGUC UCCUCCAU	1710
2216	UGAUGGAG CUGAUGAG X CGAA AGACAGGG	1403	CCCUGUCUC CUCCAUCA	1711
2219	AAAUGAUG CUGAUGAG X CGAA AGGAGACA	1404	UGUCUCCUC CAUCAUUU	1712
2223	AACCAAAU CUGAUGAG X CGAA AUGGAGGA	1405	UCCUCCAUC AUUUGGUU	1713
2226	GGAAACCA CUGAUGAG X CGAA AUGAUGGA	1406	UCCAUCAUU UGGUUUCC	1714
2227	AGGAAACC CUGAUGAG X CGAA AAUGAUGG	1407	CCAUCAUUU GGUUUCCU	1715
2231	CAAGAGGA CUGAUGAG X CGAA ACCAAAUG	1408	CAUUUGGUU UCCUCUUG	1716
2232	CCAAGAGG CUGAUGAG X CGAA AACCAAAU	1409	AUUUGGUU CCUCUUGG	1717
2233	GCCAAGAG CUGAUGAG X CGAA AAACCAAA	1410	UUUGGUUUC CUCUUGGC	1718
2236	AAAGCCAA CUGAUGAG X CGAA AGGAAACC	1411	GGUUUCCUC UGGGUUU	1719
2238	CAAAGGCC CUGAUGAG X CGAA AGAGGAAA	1412	UUUCCUCUU GGCUUUGG	1720
2243	UAUCCCCA CUGAUGAG X CGAA AGCCAAGA	1413	UCUUGGUU UGGGGAU	1721
2244	GUAUCCCC CUGAUGAG X CGAA AAGCCAAG	1414	CUUGGUUU GGGGAUAC	1722
2251	UUUAGAAG CUGAUGAG X CGAA AUCCCCAA	1415	UUGGGGAUA CUUCUAAA	1723
2254	AAAAUUUAG CUGAUGAG X CGAA AGUAUCC	1416	GGGAUACUU CUAAAAUUU	1724
2255	AAAAUUUUA CUGAUGAG X CGAA AAGUAUCC	1417	GGAUACUUC UAAAAUUU	1725
2257	CCAAAAUU CUGAUGAG X CGAA AGAAGUAU	1418	AUACUUCUA AAAUUUUGG	1726
2261	GUUCCCCA CUGAUGAG X CGAA AUUUAGAA	1419	UUCUAAUUU UGGGGAGC	1727
2262	AGCUCCCCA CUGAUGAG X CGAA AAAAAAGA	1420	UCUAAUUU UGGGAGCU	1728

Pos	RZ	SEQ ID. No.	Substrate	SEQ ID. No.
2263	GAGCUCCC CUGAUGAG X CGAA AAAUUUAG	1421	CUAAAUUUU GGGAGCUC	1729
2271	AGAUGGAG CUGAUGAG X CGAA AGCUCCCA	1422	UGGGAGCUC CUCCAUU	1730
2274	UGGAGAUG CUGAUGAG X CGAA AGGAGCUC	1423	GAGCUCCUC CAUCUCCA	1731
2278	CCAUJUGGA CUGAUGAG X CGAA AUGGAGGA	1424	UCCUCCAUC UCCAAUUGG	1732
2280	AGCCAUUG CUGAUGAG X CGAA AGAUGGAG	1425	CUCCAUUC CAAUGGCU	1733
2294	CUGCCACA CUGAUGAG X CGAA AUCCCAGC	1426	GCUGGGAUU UGUGGCAG	1734
2295	CCUGCCAC CUGAUGAG X CGAA AAUCCAG	1427	CUGGGAUUU GUGGCAGG	1735
2307	CUGAGUGG CUGAUGAG X CGAA AUCCCUGC	1428	GCAGGGAUU CCACUCAG	1736
2308	UCUGAGUG CUGAUGAG X CGAA AAUCCUG	1429	CAGGGAUUC CACUCAGA	1737
2313	GAGGUUCU CUGAUGAG X CGAA AGUGGAAU	1430	AUUCCACUC AGAACCUU	1738
2321	AUUCCAGA CUGAUGAG X CGAA AGGUUCUG	1431	CAGAACCUU UCUGGAAU	1739
2323	AAAUCCA CUGAUGAG X CGAA AGAGGUUC	1432	GAACCUCUC UGGAAUUU	1740
2330	CAGGCACA CUGAUGAG X CGAA AUUCCAGA	1433	UCUGGAAUU UGUGCCUG	1741
2331	UCAGGCAC CUGAUGAG X CGAA AAUCCAG	1434	CUGGAAUUU GUGCCUGA	1742
2347	UCCAGUGG CUGAUGAG X CGAA AGGCACAU	1435	AUGUGCCUU CCACUGGA	1743
2348	AUCCAGUG CUGAUGAG X CGAA AAGGCACA	1436	UGUGCCUUC CACUGGAU	1744
2357	AACCCCAA CUGAUGAG X CGAA AUCCAGUG	1437	CAUGGGAUU UGGGGUUU	1745
2358	GAACCCCA CUGAUGAG X CGAA AAUCCAGU	1438	ACUGGGAUU UGGGUUC	1746
2359	GGAACCCC CUGAUGAG X CGAA AAAUCCAG	1439	CUGGAAUUU GGGGUUCC	1747
2365	GUGCUGGG CUGAUGAG X CGAA ACCCCAAA	1440	UUUGGGGUU CCCAGCAC	1748
2366	GGUGCUGG CUGAUGAG X CGAA AACCCCAA	1441	UUGGGGUUC CCAGCACC	1749
2385	CCCCCCAA CUGAUGAG X CGAA AUCCACAU	1442	AUGUGGAUU UGGGGGGG	1750
2386	ACCCCCCA CUGAUGAG X CGAA AAUCCACA	1443	UGUGGAAUU UGGGGGGU	1751
2387	GACCCCCC CUGAUGAG X CGAA AAAUCCAC	1444	GUGGAAUUU GGGGGGUU	1752
2395	ACAAAAGG CUGAUGAG X CGAA ACCCCCCA	1445	UGGGGGGUUC CCUUUUGU	1753
2399	AGACACAA CUGAUGAG X CGAA AGGGACCC	1446	GGGUCCCCUU UUGUGUCU	1754
2400	GAGACACA CUGAUGAG X CGAA AAGGGACC	1447	GGUCCCCUUU UGUGUCUC	1755
2401	GGAGACAC CUGAUGAG X CGAA AAAGGGAC	1448	GUCCCCUUU UGUGUCUCC	1756
2406	GCGGGGGA CUGAUGAG X CGAA ACACAAAA	1449	UUUUGUGUC UCCCCCGC	1757
2408	UGGCGGGG CUGAUGAG X CGAA AGACACAA	1450	UUGUGUCUC CCCCCGCCA	1758
2418	AGUCCUUG CUGAUGAG X CGAA AUGGCGGG	1451	CCCGCCAUU CAAGGACU	1759
2419	GAGUCCUU CUGAUGAG X CGAA AAUGGCGG	1452	CCGCCAUUC AAGGACUC	1760
2427	AAAGAGAG CUGAUGAG X CGAA AGUCCUUG	1453	CAAGGACUC CUCUCUUU	1761
2430	AAGAAAGA CUGAUGAG X CGAA AGGAGUCC	1454	GGACUCCUC UCUUUCUU	1762
2432	UGAAGAAA CUGAUGAG X CGAA AGAGGAGU	1455	ACUCCUCUC UUUCUUC	1763
2434	GGUGAAGA CUGAUGAG X CGAA AGAGGAGA	1456	UCCUCUCUU UCUCUACC	1764
2435	UGGUGAAG CUGAUGAG X CGAA AAGAGAGG	1457	CCUCUCUUU CUUCACCA	1765
2436	UUGGUGAA CUGAUGAG X CGAA AAAGAGAG	1458	CUCUCUUUC UUCACCAA	1766
2438	UCUUGGUG CUGAUGAG X CGAA AGAAAGAG	1459	CUCUUUCUU CACCAAGA	1767
2439	UUCUUGGU CUGAUGAG X CGAA AAGAAAGA	1460	UCUUUCUUC ACCAAGAA	1768

Where "X" represents stem II region of a HH ribozyme (Hertel et al., 1992 Nucleic Acids Res. 20: 3252). The length of stem II may be  $\geq$  2 base-pairs.

Table XV: Human A-rif Hairpin Ribozyme and Target Sequence

nt. Position	Ribozyme Sequence	SEQ ID. No.	Target Sequence	SEQ ID. No.
69	CACAGA AGAA GCCU ACCAGAGAAACA X GUACAUUACCUUGUA	1769	AGGGC GAC UCGUG	1841
117	CGCUAC AGAA GCGG ACCAGAGAAACA X GUACAUUACCUUGUA	1770	CGGGC GCU GUAGCG	1842
120	CGCCGC AGAA GCGG ACCAGAGAAACA X GUACAUUACCUUGUA	1771	CGGCCU GUA GCGGGC	1843
151	GGCGUG AGAA GGUG ACCAGAGAAACA X GUACAUUACCUUGUA	1772	CACCU GCC CAGCCC	1844
156	AGGUGG AGAA GGGC ACCAGAGAAACA X GUACAUUACCUUGUA	1773	GCCCCA GCC CCACCU	1845
167	AAGAUG AGAA GAGG ACCAGAGAAACA X GUACAUUACCUUGUA	1774	CCUCA GCC CAUCU	1846
268	UUGUUG AGAA GGUA ACCAGAGAAACA X GUACAUUACCUUGUA	1775	UACCU GCC CAACAA	1847
296	AUCCCG AGAA GUCA ACCAGAGAAACA X GUACAUUACCUUGUA	1776	UGACU GUC CGGGAU	1848
366	CCACAC AGAA GUCC ACCAGAGAAACA X GUACAUUACCUUGUA	1777	GGACU GCU GUGUGG	1849
381	UGAUG AGAA GUAG ACCAGAGAAACA X GUACAUUACCUUGUA	1778	CUACC GAC UCAUCA	1850
410	GUCCCC AGAA GUCA ACCAGAGAAACA X GUACAUUACCUUGUA	1779	UCACU GCC UGGGAC	1851
478	AUGGUC AGAA GGAC ACCAGAGAAACA X GUACAUUACCUUGUA	1780	GUCCC GCU GACCAU	1852
481	UGCAUG AGAA GGCA ACCAGAGAAACA X GUACAUUACCUUGUA	1781	CCGCU GAC CAUGCA	1853
516	ACGCCA AGAA GAAG ACCAGAGAAACA X GUACAUUACCUUGUA	1782	CUUCA GCC UGGCGU	1854
537	ACUURA AGAA GAAG ACCAGAGAAACA X GUACAUUACCUUGUA	1783	CUUCU GCU UUAAGU	1855
550	CCAUGG AGAA GAAA ACCAGAGAAACA X GUACAUUACCUUGUA	1784	UUUCU GUU CCAUGG	1856
564	UUUGGC AGAA GAAG ACCAGAGAAACA X GUACAUUACCUUGUA	1785	CUUCC GUU GCCAAGA	1857
620	AACACA AGAA GUGG ACCAGAGAAACA X GUACAUUACCUUGUA	1786	CCACA GUC UGUGUU	1858
652	UGGURG AGAA GUUG ACCAGAGAAACA X GUACAUUACCUUGUA	1787	CAACA GUU CUACCA	1859
714	UCAGGG AGAA GUUC ACCAGAGAAACA X GUACAUUACCUUGUA	1788	GAACC GCC CCCUGA	1860
750	UGGGGG AGAA GGGG ACCAGAGAAACA X GUACAUUACCUUGUA	1789	UCCCA GCC CCCGCA	1861
794	GGCUGG AGAA GGGG ACCAGAGAAACA X GUACAUUACCUUGUA	1790	UCCCCU GCC CCAGCC	1862
825	ACGGGG AGAA GAUG ACCAGAGAAACA X GUACAUUACCUUGUA	1791	CAUCC GCU CCACGU	1863
866	CAUGGG AGAA GUUG ACCAGAGAAACA X GUACAUUACCUUGUA	1792	CCACG GCC CCCAUG	1864
892	CCAGUG AGAA GGAA ACCAGAGAAACA X GUACAUUACCUUGUA	1793	AUCCA GCU CACUGG	1865
917	GGCAGC AGAA GUGC ACCAGAGAAACA X GUACAUUACCUUGUA	1794	GCACU GAU GCUGC	1866
923	ACUACC AGAA GCAU ACCAGAGAAACA X GUACAUUACCUUGUA	1795	AUGCU GCU GGAGU	1867
927	CUCUAC AGAA GGCA ACCAGAGAAACA X GUACAUUACCUUGUA	1796	UGGCC GUA GUAGG	1868
969	UGGCUG AGAA GGCG ACCAGAGAAACA X GUACAUUACCUUGUA	1797	CCCCA GCC CAGCCA	1869
1049	CUUGUC AGAA GCCA ACCAGAGAAACA X GUACAUUACCUUGUA	1798	UGGCC GAU GACAAG	1870
1126	UUCAGC AGAA GCAC ACCAGAGAAACA X GUACAUUACCUUGUA	1799	GUGCA GCU GCUGAA	1871
1129	CUCUUC AGAA GGUG ACCAGAGAAACA X GUACAUUACCUUGUA	1800	CAGCU GCU GAAGAG	1872
1219	GGCUGUG AGAA GGAA ACCAGAGAAACA X GUACAUUACCUUGUA	1801	UCCCA GCC CACAGC	1873
1226	CUGCUC AGAA GUUG ACCAGAGAAACA X GUACAUUACCUUGUA	1802	CCACA GCU GAGCAG	1874
1297	CCCAUA AGAA GCAA ACCAGAGAAACA X GUACAUUACCUUGUA	1803	UUGCU GUV UAGGG	1875
1318	AAUCCC AGAA GGU ACCAGAGAAACA X GUACAUUACCUUGUA	1804	ACCCG GCC GGGAU	1876
1359	GGUAGA AGAA GGAG ACCAGAGAAACA X GUACAUUACCUUGUA	1805	CUCCA GCC UCURACC	1877
1408	UGGAUG AGAA GGAC ACCAGAGAAACA X GUACAUUACCUUGUA	1806	GUCCA GCU CAUCGA	1878

nt. Position	Ribozyme Sequence	SEQ ID. No.	Target Sequence	SEQ ID. No.
1429	UGGCA AGAA GCG ACCAGAGAAACA X GUACAUUACCUGGUAA	1807	CGGCA GAC UGCCCA	1879
1433	GCCCCUG AGAA GUCU ACCAGAGAAACA X GUACAUUACCUGGUAA	1808	AGACU GCC CAGGGC	1880
1437	UCCUAAG AGAA GGC ACCAGAGAAACA X GUACAUUACCUGGUAA	1809	GCCCA GCC CUIUGGA	1881
1576	CCUGAG AGAA GCU ACCAGAGAAACA X GUACAUUACCUGGUAA	1810	GAGCA GCC CUCAGG	1882
1588	CCUGAG AGAA GCU ACCAGAGAAACA X GUACAUUACCUGGUAA	1811	UGGCA GCU GAGGUU	1883
1616	CACCUU AGAA GCU ACCAGAGAAACA X GUACAUUACCUGGUAA	1812	GAUCC GUA UGCAGG	1884
1629	CCUGCA AGAA GAUC ACCAGAGAAACA X GUACAUUACCUGGUAA	1813	CUACA GCU UCCAGU	1885
1653	ACUGGA AGAA GUAG ACCAGAGAAACA X GUACAUUACCUGGUAA	1814	AGUCA GAC GUCUAU	1886
1664	AUAGAC AGAA GACU ACCAGAGAAACA X GUACAUUACCUGGUAA	1815	UCACU GCC UUACAG	1887
1714	CUUGUA AGAA GUGA ACCAGAGAAACA X GUACAUUACCUGGUAA	1816	UGGCU GCC GUGACC	1888
1734	GGUCAC AGAA GCCA ACCAGAGAAACA X GUACAUUACCUGGUAA	1817	GACCA GAU UAUUCU	1889
1744	AAGAUU AGAA GGUIC ACCAGAGAAACA X GUACAUUACCUGGUAA	1818	UAUCU GUC CCCGGA	1890
1774	UCCGGG AGAA GAUA ACCAGAGAAACA X GUACAUUACCUGGUAA	1819	CCCCG GAC CUCAGC	1891
1781	GCUGAG AGAA GGGG ACCAGAGAAACA X GUACAUUACCUGGUAA	1820	CAACU GCC CCAAGG	1892
1806	CCUUGG AGAA GUUG ACCAGAGAAACA X GUACAUUACCUGGUAA	1821	CGCCU GCU GUCUGA	1893
1828	UCAGAC AGAA GGGG ACCAGAGAAACA X GUACAUUACCUGGUAA	1822	CUGCU GUC UGACUG	1894
1831	CAGUCA AGAA GCG AGCAG ACCAGAGAAACA X GUACAUUACCUGGUAA	1823	UGUCU GAC UGCCUC	1895
1835	GAGGCA AGAA GACA ACCAGAGAAACA X GUACAUUACCUGGUAA	1824	UGACU GCC UCAAGU	1896
1839	ACUUGA AGAA GUGA ACCAGAGAAACA X GUACAUUACCUGGUAA	1825	GAGCG GCC CCUCU	1897
1864	AAGAGG AGAA GCUIC ACCAGAGAAACA X GUACAUUACCUGGUAA	1826	CCCCA GAU CCUGGC	1898
1879	GCCAGG AGAA GGGG ACCAGAGAAACA X GUACAUUACCUGGUAA	1827	GAGCU GCU GCAACG	1899
1900	CGUUGC AGAA GCUIC ACCAGAGAAACA X GUACAUUACCUGGUAA	1828	AGGCC GAU GAGUUG	1900
1967	CAUCUC AGAA GCCU ACCAGAGAAACA X GUACAUUACCUGGUAA	1829	UGCCU GCC UGCCUA	1901
1979	UAGGCA AGAA GGC ACCAGAGAAACA X GUACAUUACCUGGUAA	1830	UGCCU GCC UACUCA	1902
1983	UGAGUA AGAA GGC ACCAGAGAAACA X GUACAUUACCUGGUAA	1831	GGCGA GCC CGCCU	1903
1997	AAGGCG AGAA GGGG ACCAGAGAAACA X GUACAUUACCUGGUAA	1832	AGCCC GCC UUGUGC	1904
2001	GCACAA AGAA GGGU ACCAGAGAAACA X GUACAUUACCUGGUAA	1833	GCCCC GCC CAAGGC	1905
2020	GGCUUUG AGAA GGGC ACCAGAGAAACA X GUACAUUACCUGGUAA	1834	UCUCA GCC CUCAC	1906
2047	GUGGAG AGAA GAGA ACCAGAGAAACA X GUACAUUACCUGGUAA	1835	UCUCU GCC CUGAUG	1907
2097	CAUCAG AGAA GAGA ACCAGAGAAACA X GUACAUUACCUGGUAA	1836	GGCCU GAU GCUGCC	1908
2102	GGCAGC AGAA GGGC ACCAGAGAAACA X GUACAUUACCUGGUAA	1837	AUGCU GCC UCAGGA	1909
2108	UCCUGA AGAA GCAU ACCAGAGAAACA X GUACAUUACCUGGUAA	1838	UICCA GUO CUUCUG	1910
2167	CAGAAG AGAA GGAA ACCAGAGAAACA X GUACAUUACCUGGUAA	1839	CCCCU GUIC UCCUC	1911
2211	GGAGGA AGAA GGG ACCAGAGAAACA X GUACAUUACCUGGUAA	1840	UGCCU GAU GUGCCU	1912
2337	AGGCAC AGAA GGCA ACCAGAGAAACA X GUACAUUACCUGGUAA			

Where "X" represents stem IV region of a Hairpin ribozyme. The length of stem IV may be 2 base-pairs.

Table XVI: Hammerhead Ribozyme Sites for B-raf

nt. Position	Ribozyme	SEQ ID. No.	Substrate	SEQ ID. No.
17	CGGGCGGG CUGAUGAG X CGAA AGGGGCC	1913	GGCCCCCUC CCCGCCG	2354
38	CGGGGCC CUGAUGAG X CGAA AGCGGCCG	1914	CGGCGCUC GGGCCCCG	2355
50	AUAACCAGA CUGAUGAG X CGAA AGCCGGGG	1915	CCCCGGCUC UCGGUUAU	2356
52	UUUAUAAACC CUGAUGAG X CGAA AGAGCCGG	1916	CCGGCUCUC GGUUAUAA	2357
56	CAUCUUAU CUGAUGAG X CGAA ACCGAGAG	1917	CUCUCGGUU AUAAAUG	2358
57	CCAUCUUA CUGAUGAG X CGAA AACCGAGA	1918	UCUCGGUUA UAAGAUGG	2359
59	CGCCAUCU CUGAUGAG X CGAA AUAAACCA	1919	UCGGGUUAUA AGAUGGCG	2360
113	GUUGAACAC CUGAUGAG X CGAA AGCCUGGC	1920	GCCAGGCUC UGUUCAAC	2361
117	CCCCGUUG CUGAUGAG X CGAA ACAGAGCC	1921	GGCUCUGUU CAACGGGG	2362
118	UCCCCGUU CUGAUGAG X CGAA AACAGAGC	1922	GCUCUGUUC AACGGGGA	2363
165	CAGCCGAA CUGAUGAG X CGAA AGGCCGCG	1923	CGCGGCCUC UUCGGCUG	2364
167	CGCAGCCG CUGAUGAG X CGAA AGAGGCCG	1924	CGGCCUCUU CGGCUGCG	2365
168	CCGCAGCC CUGAUGAG X CGAA AAGAGGCC	1925	GGCCUCUUC GGCUGCGG	2366
187	UCCUCCGG CUGAUGAG X CGAA AUGGCAGG	1926	CCUGCCAUAU CCGGAGGA	2367
188	CUCCUCCG CUGAUGAG X CGAA AAUGGCAG	1927	CUGCCAUUC CGGAGGAG	2368
206	UUGUUUGA CUGAUGAG X CGAA AUUCCACA	1928	UGUGGAAUA UCAAACAA	2369
208	AUUGUUUU CUGAUGAG X CGAA AUAUUCCA	1929	UGGAAUAUC AAACAAAU	2370
220	GUCAACUU CUGAUGAG X CGAA AUCAUUUG	1930	CAAUGAUU AAGUUGAC	2371
221	UGUCAACU CUGAUGAG X CGAA AAUCAUUA	1931	AAAUGAUUA AGJUGACA	2372
225	CCUGUGUC CUGAUGAG X CGAA ACUUAUC	1932	GAUUAAGUU GACACAGG	2373
239	GGCCUCUA CUGAUGAG X CGAA AUGUUCU	1933	AGGAACAUU UAGAGGCC	2374
241	AGGGCCUC CUGAUGAG X CGAA AUAUUUC	1934	GAACAUUA GAGGCCU	2375
250	UUGUCCAA CUGAUGAG X CGAA AGGGCCUC	1935	GAGGCCUA UJGGACAA	2376
252	AUUGUCC CUGAUGAG X CGAA AUAGGCC	1936	GGCCCUAUU GGACAAAU	2377
261	CCCCACCA CUGAUGAG X CGAA AUUUGUCC	1937	GGACAAAUU UGGUGGGG	2378
262	UCCCCACC CUGAUGAG X CGAA AAUUUGUC	1938	GACAAAUU GGUGGGGA	2379
275	UGGUGGGAU CUGAUGAG X CGAA AUGCUCCC	1939	GGGAGGCAUA AUCCACCA	2380
278	UGAUGGUG CUGAUGAG X CGAA AUUAAGCU	1940	AGCAUAAUC CACCAUCA	2381
285	GAUUAUUAU CUGAUGAG X CGAA AUGGUGGA	1941	UCCACCAUC AAAUAUAC	2382
289	UCCAGAUA CUGAUGAG X CGAA AUUGAUGG	1942	CCAUCAUUA UAUCUGGA	2383
291	CCUCCAGA CUGAUGAG X CGAA AUUUGAU	1943	AUCAAUUAU UCUGGAGG	2384
293	GGCCUCCA CUGAUGAG X CGAA AUUAUUG	1944	CAAUAUAUC UGGAGGCC	2385
303	AUUCUUCA CUGAUGAG X CGAA AGGCCUCC	1945	GGAGGCCUA UGAAGAAU	2386
312	UGCUGGUG CUGAUGAG X CGAA AUUCUUC	1946	UGAAGAAUA CACCAGCA	2387
325	AGUGCAUC CUGAUGAG X CGAA AGCUUGCU	1947	AGCAAGCUA GAUGCACU	2388
334	CUUUGUUG CUGAUGAG X CGAA AGUGCAUC	1948	GAUGCACUC CAACAAAG	2389
354	AUUCCAAU CUGAUGAG X CGAA ACUGUUGU	1949	ACAACAGUU AUUGGAAU	2390
355	GAUUCCAA CUGAUGAG X CGAA AACUGUUG	1950	CAACAGUUA UGGAAUC	2391
357	GAGAUUCC CUGAUGAG X CGAA AUUACUGU	1951	ACAGUUAU GGAAUCUC	2392
363	UCCCCAGA CUGAUGAG X CGAA AUUCCAAU	1952	AUUGGAAUC UCUGGGGA	2393
365	GUUCCCCA CUGAUGAG X CGAA AGAUUCCA	1953	UGGAAUCUC UGGGGAAC	2394
383	AACAGAAA CUGAUGAG X CGAA AUCAGUUC	1954	GAACUGAUU UUUCUGUU	2395
384	AAACAGAA CUGAUGAG X CGAA AAUCAGUU	1955	AACUGAUU UUCUGUUU	2396
385	GAAACAGA CUGAUGAG X CGAA AAAUCAGU	1956	ACUGAUUUU UCUGUUUC	2397
386	AGAAACAG CUGAUGAG X CGAA AAAAUUCAG	1957	CUGAUUUUU CUGUUUCU	2398
387	UAGAAACA CUGAUGAG X CGAA AAAAUCA	1958	UGAUUUUUUC UGUUUCUA	2399
391	GAGCUAGA CUGAUGAG X CGAA ACAGAAAA	1959	UUUUUCUGUU UCUAGCUC	2400
392	AGAGCUAG CUGAUGAG X CGAA AACAGAAA	1960	UUUCUGUUU CUAGCUCU	2401
393	CAGAGCUA CUGAUGAG X CGAA AAACAGAA	1961	UUCUGUUUC UAGCUCUG	2402
395	UGCAGAGC CUGAUGAG X CGAA AGAAACAG	1962	CUGUUUCUA GCUCUGCA	2403
399	UUGAUGCA CUGAUGAG X CGAA AGCUAGAA	1963	UUCUAGCUC UGCAUCAA	2404
405	UAUCCAUU CUGAUGAG X CGAA AUGCAGAG	1964	CUCUGCAUC AAUGGAAU	2405
413	UGUAACGG CUGAUGAG X CGAA AUCCAUUG	1965	CAAUGGAAU CCGGUUACA	2406

nt. Position	Rib zyme	SEQ ID. N.	Substrate	SEQ ID. No.
418	GAAGAUGU CUGAUGAG X CGAA ACGGUUAUC	1966	GAUACCGUU ACAUCUUC	2407
419	AGAAGAUG CUGAUGAG X CGAA AACGGUAU	1967	AUACCGUUA CAUCUUCU	2408
423	AGGAAGAA CUGAUGAG X CGAA AUGUAACG	1968	CGUUACAUC UUCUUCU	2409
425	AGAGGAAG CUGAUGAG X CGAA AGAUGUAA	1969	UUACAUUUU CUUCCUCU	2410
426	AAGAGGAA CUGAUGAG X CGAA AAGAUGUA	1970	UACAUUUC UUCCUCUU	2411
428	AGAAGAGG CUGAUGAG X CGAA AGAAGAUG	1971	CAUCUUCUU CCUCUUCU	2412
429	UAGAAGAG CUGAUGAG X CGAA AAGAAGAU	1972	AUCUUUCUUC CUCUUCUA	2413
432	GGCUAGAA CUGAUGAG X CGAA AGGAAGAA	1973	UUCUUCUC UUCUAGCC	2414
434	AAGGCUAG CUGAUGAG X CGAA AGAGGAAG	1974	CUUCCUCUU CUAGCCUU	2415
435	AAAGGCUA CUGAUGAG X CGAA AAGAGGAA	1975	UUCCUCUUC UAGCCUUU	2416
437	UGAAAGGC CUGAUGAG X CGAA AGAAGAGG	1976	CCUCUUCUA GCCUUUCA	2417
442	AGCACUGA CUGAUGAG X CGAA AGGCUAGA	1977	UCUAGCCUU UCAGUGCU	2418
443	UAGCACUG CUGAUGAG X CGAA AAGGCUAG	1978	CUAGCCUUU CAGUGCUA	2419
444	GUAGCACU CUGAUGAG X CGAA AAAGGCUA	1979	UAGCCUUUC AGUGCUAC	2420
451	GAUGAAGG CUGAUGAG X CGAA AGCACUGA	1980	UCAGUGCUA CCUUCAUC	2421
455	AAGAGAUG CUGAUGAG X CGAA AGGUAGCA	1981	UGCUACCUU CAUCUUU	2422
456	AAAGAGAU CUGAUGAG X CGAA AAGGUAGC	1982	GCUACCUUC AUCUCUUU	2423
459	CUGAAAGA CUGAUGAG X CGAA AUGAAGGU	1983	ACCUUCAUC UCUUUCAG	2424
461	AACUGAAA CUGAUGAG X CGAA AGAUGAAG	1984	CUUCAUCUC UUUCAGUU	2425
463	AAAACUGA CUGAUGAG X CGAA AGAGAUGA	1985	UCAUCUUU UCAGUUUU	2426
464	AAAAACUG CUGAUGAG X CGAA AAGAGAUG	1986	CAUCUUUU CAGUUUUU	2427
465	AAAAACU CUGAUGAG X CGAA AAAGAGAU	1987	AUCUCUUUC AGUUUUUUC	2428
469	UUUUGAAA CUGAUGAG X CGAA ACUGAAAG	1988	CUUUCAGUU UUCAAAA	2429
470	AUUUUGAA CUGAUGAG X CGAA AACUGAAA	1989	UUUCAGUUU UUCAAAAU	2430
471	GAUUUUGA CUGAUGAG X CGAA AAACUGAA	1990	UUCAGUUUU UCAAAAU	2431
472	GGAUUUUG CUGAUGAG X CGAA AAAACUGA	1991	UCAGUUUUU CAAAUC	2432
473	GGGAUUUU CUGAUGAG X CGAA AAAACUG	1992	CAGUUUUUC AAAAUCC	2433
479	AUCUGUGG CUGAUGAG X CGAA AUUUUGAA	1993	UUCAAAUC CCACAGAU	2434
510	UUUGUGGU CUGAUGAG X CGAA ACUUGGG	1994	CCCCAAGUC ACCACAAA	2435
524	UCUAACGA CUGAUGAG X CGAA AGGUUUUU	1995	AAAAACCUA UCGUUUAGA	2436
526	ACUCUAC CUGAUGAG X CGAA AUAGGUUU	1996	AAACCUAUC GUUAGAGU	2437
529	AAGACUCU CUGAUGAG X CGAA ACGAUAGG	1997	CCUAUCGUU AGAGUCUU	2438
530	GAAGACUC CUGAUGAG X CGAA AACGAUAG	1998	CUAUCGUU GAGUCUUC	2439
535	GGCAGGAA CUGAUGAG X CGAA ACUCUAAC	1999	GUUAGAGUC UUCCUGCC	2440
537	UGGGCAGG CUGAUGAG X CGAA AGACUCUA	2000	UAGAGUCUU CCUGCCCA	2441
538	UUGGGCAG CUGAUGAG X CGAA AAGACUCU	2001	AGAGUCUUC CUGCCCAA	2442
565	CUUGCAGG CUGAUGAG X CGAA ACCACUGU	2002	ACAGUGGUU CCUGCAAG	2443
583	CGGACUGU CUGAUGAG X CGAA ACUCCACA	2003	UGUGGAGUU ACAGUCG	2444
584	UCGGACUG CUGAUGAG X CGAA AACUCCAC	2004	GUGGAGUUU CAGUCCGA	2445
589	CUGUCUCG CUGAUGAG X CGAA ACUGUAAC	2005	GUUACAGUC CGAGACAG	2446
599	UUUCUUUA CUGAUGAG X CGAA ACUGUCU	2006	GAGACAGUC UAAAGAAA	2447
601	GUUUUCUU CUGAUGAG X CGAA AGACUGUC	2007	GACAGUCUA AGAAAGC	2448
626	UGGGAUUA CUGAUGAG X CGAA ACCUCUCA	2008	UGAGAGGUU UAAUCCCA	2449
628	UCUGGGAU CUGAUGAG X CGAA AGACCUCU	2009	AGAGGUCUA AUCCCA	2450
631	CACUCUGG CUGAUGAG X CGAA AUUAGACC	2010	GGUCUAAUC CCAGAGUG	2451
649	AUUCUGUA CUGAUGAG X CGAA ACAGCACA	2011	UGUGCUGUU UACAGAAU	2452
650	AAUUCUGU CUGAUGAG X CGAA AACAGCAC	2012	GUGCUGUUU ACAGAAUU	2453
651	GAUUUCUG CUGAUGAG X CGAA AAACAGCA	2013	UGCUGUUUA CAGAAUUC	2454
658	CCAUCUUG CUGAUGAG X CGAA AUUCUGUA	2014	UACAGAAUU CAGGAUGG	2455
659	UCCAUCU CUGAUGAG X CGAA AAUUCUGU	2015	ACAGAAUUC AGGAUGGA	2456
682	UCCCAACC CUGAUGAG X CGAA AUUGGUUU	2016	AAACCAAUU GGUUGGGA	2457
686	AGUGUCCC CUGAUGAG X CGAA ACCAAUUG	2017	CAAUUGGUU GGGACACU	2458
698	CCAGGAAA CUGAUGAG X CGAA AUCAGUGU	2018	ACACUGUA UUCCUGG	2459
700	AGCCAGGA CUGAUGAG X CGAA AUAUCAGU	2019	ACUGAUAUU UCCUGGCC	2460
701	AAGCCAGG CUGAUGAG X CGAA AAAUACAG	2020	CUGAUUUU CCUGGCUU	2461

nt. Positi n	Ribozyme	SEQ ID. N	Substrate	SEQ ID. No.
702	UAAGCCAG CUGAUGAG X CGAA A <sub>n</sub> UAUCA	2021	UGAUAUUUC CUGGCUUA	2462
709	UCUCCAGU CUGAUGAG X CGAA AGCCAGGA	2022	UCCUGGCUU ACUGGAGA	2463
710	UUCUCCAG CUGAUGAG X CGAA AAGCCAGG	2023	CCUGGCUUA CUGGAGAA	2464
723	CCACAAUGC CUGAUGAG X CGAA AUUCJUCU	2024	AGAAGAAUU GCAUGUGG	2465
738	CAUUCUCC CUGAUGAG X CGAA ACACUUCC	2025	GGAAGUGUU GGAGAAUG	2466
748	GUAAGUGG CUGAUGAG X CGAA ACAUUCUC	2026	GAGAAUGUU CCACUUAC	2467
749	UGUAAGUG CUGAUGAG X CGAA AACAUUCU	2027	AGAAUGUUC CACUUACA	2468
754	UGUGUUGU CUGAUGAG X CGAA AGUGGAAC	2028	GUUCCACUU ACAACACA	2469
755	GUGUGUUG CUGAUGAG X CGAA AAGUGGAA	2029	UUCCACUU CAACACAC	2470
768	UUCGUACA CUGAUGAG X CGAA AGUUGUGU	2030	ACACAAACUU UGUACGAA	2471
769	UUUCGUAC CUGAUGAG X CGAA AAGUUGUG	2031	CACAACUUU GUACGAAA	2472
772	GUUUUUUCG CUGAUGAG X CGAA ACAAAGUU	2032	AACUUUGUA CGAAAAAC	2473
783	AGGUGAAA CUGAUGAG X CGAA ACGUUUUU	2033	AAAAACGUU UUUCACCU	2474
784	AAGGUGAA CUGAUGAG X CGAA AACGUUUU	2034	AAAACGUUU UUCACCUU	2475
785	UAAGGUGA CUGAUGAG X CGAA AAACGUUU	2035	AAACGUUUU UCACCUUA	2476
786	CUAAGGUG CUGAUGAG X CGAA AAAACGUU	2036	AACGUUUUUU CACCUUAG	2477
787	GCUAAGGU CUGAUGAG X CGAA AAAAACGU	2037	ACGUUUUUUC ACCUUAGC	2478
792	AAAAUGCU CUGAUGAG X CGAA AGGUGAAA	2038	UUUCACCUU AGCAUUUU	2479
793	CAAAAUGC CUGAUGAG X CGAA AAGGUGAA	2039	UUCACCUUA GCAJUUUG	2480
798	AGUCACAA CUGAUGAG X CGAA AUGCUAAG	2040	CUUAGCAUU UUGUGACU	2481
799	AAGUCACA CUGAUGAG X CGAA AAUGCUAA	2041	UUAGCAUUU UGUGACUU	2482
800	AAAGUCAC CUGAUGAG X CGAA AAAUGCUA	2042	UAGCAUUUU GUGACUUU	2483
807	UUCGACAA CUGAUGAG X CGAA AGUCACAA	2043	UUGUGACUU UUGUCGAA	2484
808	UUUCGACA CUGAUGAG X CGAA AAGUCACA	2044	UGUGACUUU UGUCGAAA	2485
809	CUUUCGAC CUGAUGAG X CGAA AAAGUCAC	2045	GUGACUUUU GUCGAAAG	2486
812	CAGCUUUC CUGAUGAG X CGAA ACAAAAGU	2046	ACUUUUGUC GAAAGCUG	2487
823	CCCUGGAA CUGAUGAG X CGAA AGCAGCUU	2047	AAGCUGCUU UUCCAGGG	2488
824	ACCCUGGA CUGAUGAG X CGAA AAGCAGCU	2048	AGCUGCUUU UCCAGGGU	2489
825	ACCCUGG CUGAUGAG X CGAA AAAGCAGC	2049	GCUGCUUUU CCAGGGUU	2490
826	AAACCCUG CUGAUGAG X CGAA AAAAGCAG	2050	CUGCUUUUC CAGGGUUU	2491
833	ACAGCGGA CUGAUGAG X CGAA ACCCUGGA	2051	UCCAGGGUU UCCGCUGU	2492
834	GACAGCGG CUGAUGAG X CGAA AACCCUGG	2052	CCAGGGUUU CCGCUGUC	2493
835	UGACAGCG CUGAUGAG X CGAA AAACCCUG	2053	CAGGGUUUC CGCUGUCA	2494
842	ACAUGUUU CUGAUGAG X CGAA ACAGCGGA	2054	UCCGCUGUC AAACAUGU	2495
854	AAAAUUUAU CUGAUGAG X CGAA ACCACAUG	2055	CAUGUGGUU AUAAAUUU	2496
855	GAAAAUUA CUGAUGAG X CGAA AACCAACAU	2056	AUGUGGUUA UAAAUUUC	2497
857	GUGAAAAU CUGAUGAG X CGAA AUUACCCAC	2057	GUGGUUAUA AUUUCAC	2498
861	GCUGGUGA CUGAUGAG X CGAA AUUUUAUA	2058	UUUAUUUU UCACCAAC	2499
862	CGCUGGUG CUGAUGAG X CGAA AUUUUAUA	2059	UUAUAAAUUU CACCAGCG	2500
863	ACGCUGGU CUGAUGAG X CGAA AAAUUUAU	2060	AUAAAUUUC ACCAGCGU	2501
872	UGUACUAC CUGAUGAG X CGAA ACGCUGGU	2061	ACCAAGCGU GUAGUACA	2502
875	UUCUGUAC CUGAUGAG X CGAA ACAACCGU	2062	AGCGUUGUA GUACAGAA	2503
878	AACUUCUG CUGAUGAG X CGAA ACUACAAC	2063	GUUGUAGUA CAGAAGUU	2504
886	AUCAGUGG CUGAUGAG X CGAA AAUCUCUG	2064	ACAGAAGUU CCACUGAU	2505
887	CAUCAGUG CUGAUGAG X CGAA AACUUCUG	2065	CAGAAGUUC CACUGAUG	2506
901	UCAUAAA CUGAUGAG X CGAA ACACACAU	2066	AUGUGUGUU AAUUAUGA	2507
902	GUCAUAAA CUGAUGAG X CGAA AACACACA	2067	UGUGUGUUA AUUAUGAC	2508
905	UUGGUCAU CUGAUGAG X CGAA AUUAACAC	2068	GUGUAAAUA AUGACCAA	2509
906	GUUGGUCA CUGAUGAG X CGAA AAUUAACA	2069	UGUUAUUUA UGACCAAC	2510
916	AGCAAAC CUGAUGAG X CGAA AGUUGUC	2070	GACCAACUU GAUUGCU	2511
920	AAACAGCA CUGAUGAG X CGAA AUCAAGU	2071	AACUUGAUU UGCUGUUU	2512
921	CAAACAGC CUGAUGAG X CGAA AAUCAAGU	2072	ACUUGAUUU GCUGUUUG	2513
927	UGGAGACA CUGAUGAG X CGAA ACAGCAAA	2073	UUUGCUGUU UGUCUCCA	2514
928	UGGAGAC CUGAUGAG X CGAA AACAGCAA	2074	UUGCUGUUU GUCUCCAA	2515
931	AACUUGGA CUGAUGAG X CGAA ACAACAG	2075	CUGUUUGUC UCCAAGUU	2516

nt. P siti n	Rib zyme	SEQ ID. N.	Substrat	SEQ ID. N.
933	AGAACUUG CUGAUGAG X CGAA AGACAAAC	2076	GUUUGUCUC CAAGUUCU	2517
939	GUUCAAAG CUGAUGAG X CGAA ACUUGGAG	2077	CUCCAAGUU CUUUGAAC	2518
940	UGUUCAAA CUGAUGAG X CGAA AACUUGGA	2078	UCCAAGUUC UUUGAAC	2519
942	GGUGUUCUCA CUGAUGAG X CGAA AGAACUUG	2079	CAAGUUCUU UGAACACC	2520
943	UGGUGUUC CUGAUGAG X CGAA AAAAACUU	2080	AAGUUUUU GAACACCA	2521
958	UCCUGUGG CUGAUGAG X CGAA AUUGGGUG	2081	CACCCAAUA CCACAGGA	2522
975	CUGCUAAG CUGAUGAG X CGAA ACGCCUCU	2082	AGAGGCGUC CUUAGCAG	2523
978	UCUCUGCU CUGAUGAG X CGAA AGGACGCC	2083	GGCGUCCUU AGCAGAGA	2524
979	GUCUCUGC CUGAUGAG X CGAA AAGGACGC	2084	GCGUCCUUU GCAGAGAC	2525
994	CCAGAUGU CUGAUGAG X CGAA AGGGCAGU	2085	ACUGCCUA ACAUCUGG	2526
999	AUGAUCCA CUGAUGAG X CGAA AUGUUAGG	2086	CCUAACAUC UGGAUCAU	2527
1005	AAGGGGAU CUGAUGAG X CGAA AUCCAGAU	2087	AUCUGGAUC AUCCCCUU	2528
1008	CGGAAGGG CUGAUGAG X CGAA AUGAUCCA	2088	UGGAUCAUC CCCUCCG	2529
1013	GGGUGCGG CUGAUGAG X CGAA AGGGGAUG	2089	CAUCCCCUU CCGCACCC	2530
1014	CGGGUGCG CUGAUGAG X CGAA AAGGGGAU	2090	AUCCCCUUC CGCACCCG	2531
1026	UAGAGUCC CUGAUGAG X CGAA AGGCGGGU	2091	ACCCGCCUC GGACUCUA	2532
1032	GCCCCAAUA CUGAUGAG X CGAA AGUCCGAG	2092	CUCGGACUC UAUUGGGC	2533
1034	GGGCCCCA CUGAUGAG X CGAA AGAGUCCG	2093	CGGACUCUA UUGGGCCC	2534
1036	UGGGGGCCC CUGAUGAG X CGAA AUAGAGUC	2094	GACUCUAAU GGGCCCCA	2535
1048	CUGGUGAG CUGAUGAG X CGAA AUUUGGGG	2095	CCCCAAAUU CUCACCAG	2536
1049	ACUGGUGA CUGAUGAG X CGAA AAUJJUGGG	2096	CCCAAUUC UCACCAU	2537
1051	GGACUGGU CUGAUGAG X CGAA AGAAUUUG	2097	CAAAUUCUC ACCAGUCC	2538
1058	AGGAGACG CUGAUGAG X CGAA ACUGGUGA	2098	UCACCAGUC CGUCUCCU	2539
1062	UUGAAGGA CUGAUGAG X CGAA ACGGACUG	2099	CAGUCCGUC UCCUCAA	2540
1064	UUUUGAAG CUGAUGAG X CGAA AGACGGAC	2100	GUCCGUCUC CUUAAAAA	2541
1067	GGAAUUUG CUGAUGAG X CGAA AGGAGACG	2101	CGUCUCCUU CAAAUC	2542
1068	UGGAUUUU CUGAUGAG X CGAA AAGGAGAC	2102	GUCUCCUUC AAAAUCC	2543
1074	UUGGAAUG CUGAUGAG X CGAA AUUUUGAA	2103	UUCAAAUUC CAUUC	2544
1078	GGAAUUGG CUGAUGAG X CGAA AUGGAAU	2104	AAAUCCAUU CCAAUUCC	2545
1079	UGGAUUUG CUGAUGAG X CGAA AAUGGAU	2105	AAUCCAUUC CAAUCCA	2546
1084	GGCUGUGG CUGAUGAG X CGAA AUUGGAU	2106	AAUCCAAUU CCACAGCC	2547
1085	GGGCUGUG CUGAUGAG X CGAA AAUUGGAA	2107	UUCCAAUUC CACAGCCC	2548
1095	CUGGUCGG CUGAUGAG X CGAA AGGGCUGU	2108	ACAGCCUUU CCGACCAG	2549
1096	GCUGGUCG CUGAUGAG X CGAA AAGGGCUG	2109	CAGCCCUUC CGACCAGC	2550
1115	AUUUCGAU CUGAUGAG X CGAA AUCUUCAU	2110	AUGAAGAUC AUCGAAAU	2551
1118	UUGAUUUC CUGAUGAG X CGAA AUGAUUU	2111	AAGAUCAUC GAAAUCAA	2552
1124	CCCAAUUU CUGAUGAG X CGAA AUUUCGAU	2112	AUCGAAAUU AAUUGGG	2553
1128	GUUGCCCA CUGAUGAG X CGAA AUUGAUU	2113	AAAUCAAUU UGGGCAAC	2554
1129	CGUUGCCC CUGAUGAG X CGAA AAUUGAU	2114	AAUCAAAUU GGGCAACG	2555
1146	CUGAUGAG CUGAUGAG X CGAA AUCGGUCU	2115	AGACCGAUC CUCAUCAG	2556
1149	GAGCUGAU CUGAUGAG X CGAA AGGAUCGG	2116	CCGAUCCUC AUCAGCUC	2557
1152	UGGGAGCU CUGAUGAG X CGAA AUGAGAU	2117	AUCCUCAUC AGCUCCCA	2558
1157	CACAUUUG CUGAUGAG X CGAA AGCUGAUG	2118	CAUCAGCUC CCAAUGUG	2559
1169	UGUGUUUA CUGAUGAG X CGAA AUGCACAU	2119	AUGUGCAUA UAAACACA	2560
1171	AUUGUGUU CUGAUGAG X CGAA AUUUGCAC	2120	GUGCAUUA AACACAAU	2561
1180	ACAGGUUC CUGAUGAG X CGAA AUUGUGUU	2121	AAACACAAU GAACCU	2562
1189	UCAAAUUAU CUGAUGAG X CGAA ACAGGUUC	2122	GAACCUGUC AAUAUUGA	2563
1193	GUCAUCAA CUGAUGAG X CGAA AUUGACAG	2123	CUGUCAUA UUGAUGAC	2564
1195	AAGUCAUC CUGAUGAG X CGAA AUUUGAC	2124	GUCAAAUUA GAUGACUU	2565
1203	CUCUAAUC CUGAUGAG X CGAA AGUCAUCA	2125	UGAUGACUU GAUUAGAG	2566
1207	UGGUCUCU CUGAUGAG X CGAA AUCAAGUC	2126	GACUUGAUU AGAGACCA	2567
1208	UUGGUCUC CUGAUGAG X CGAA AAUCAAGU	2127	ACUUGAUUA GAGACCAA	2568
1221	CACCAACGA CUGAUGAG X CGAA AUCCUUGG	2128	CCAAGGAUU UCGUGGGUG	2569
1222	UCACCACG CUGAUGAG X CGAA AAUCCUUG	2129	CAAGGAUUU CGUGGUGA	2570
1223	AUCACCCAC CUGAUGAG X CGAA AAAUCCUU	2130	AAGGAUUC GUGGUGAU	2571

nt. Position	Ribozyme	SEQ ID. N.	Substrate	SEQ ID. No.
1239	CUGUGGUU CUGAUGAG X CGAA AUCCUCCA	2131	UGGAGGAUC AACCACAG	2572
1250	AGCAGACA CUGAUGAG X CGAA ACCUGUGG	2132	CCACAGGUU UGUCUGCU	2573
1251	UAGCAGAC CUGAUGAG X CGAA AACUGUG	2133	CACAGGUUU GUCUGCUA	2574
1254	GGGUAGCA CUGAUGAG X CGAA ACAAAACC	2134	AGGUUUGUC UGCUACCC	2575
1259	AGGGGGGG CUGAUGAG X CGAA AGCAGACA	2135	UGUCUGCUA CCCCCCCC	2576
1272	CAGGUAAU CUGAUGAG X CGAA AGGCAGGG	2136	CCCUGCCUC AUUACCU	2577
1275	AGCCAGGU CUGAUGAG X CGAA AUGAGGC	2137	UGCCUCAUU ACCUGGC	2578
1276	GAGCCAGG CUGAUGAG X CGAA AAUGAGGC	2138	GCCUCAUUA CCUGGCUC	2579
1284	UAGUUAGU CUGAUGAG X CGAA AGCCAGGU	2139	ACCUGGCUC ACUAACUA	2580
1288	ACGUUAGU CUGAUGAG X CGAA AGUGAGCC	2140	GGCUCACUA ACUAACGU	2581
1292	UUUCACGU CUGAUGAG X CGAA AGUUAGU	2141	CACUAACUA ACGUGAAA	2582
1305	UUUCUGU CUGAUGAG X CGAA AGGCUUU	2142	GAAAGCCUU ACAGAAAU	2583
1306	GAUUCUG CUGAUGAG X CGAA AAGGCUUU	2143	AAAGCCUUA CAGAAAC	2584
1314	GUCCUGGA CUGAUGAG X CGAA AUUUCUGU	2144	ACAGAAAC UCCAGGAC	2585
1316	AGGUCCUG CUGAUGAG X CGAA AGAUUUCU	2145	AGAAACUC CAGGACCU	2586
1325	UUCUCGCU CUGAUGAG X CGAA AGGUCCUG	2146	CAGGACCC AGCGAGAA	2587
1341	AUGAAGAU CUGAUGAG X CGAA ACUUCUU	2147	AAGGAAGUC AUCUUCAU	2588
1344	AGGAUGAA CUGAUGAG X CGAA AUGACUUC	2148	GAAGUCAUC UUCAUCC	2589
1346	UGAGGAUG CUGAUGAG X CGAA AGAUGACU	2149	AGUCAUCUU CAUCCUCA	2590
1347	CUGAGGAU CUGAUGAG X CGAA AAGAUGAC	2150	GUCAUCUUC AUCCUCAG	2591
1350	CUUCUGAG CUGAUGAG X CGAA AUGAAGAU	2151	AUCUUCAU CUCAGAAG	2592
1353	UGUCUUCU CUGAUGAG X CGAA AGGAUGAA	2152	UUCAUCCUC AGAAGACA	2593
1367	UUUCAUUC CUGAUGAG X CGAA AUUCCUGU	2153	ACAGGAAC GAAUGAAA	2594
1381	CGUCUACC CUGAUGAG X CGAA AGGUUUU	2154	AAAACACUU GGUAGACG	2595
1385	GUCCCCGUC CUGAUGAG X CGAA ACCAAGUG	2155	CACUUGGUU GACGGGAC	2596
1395	CAUCACUC CUGAUGAG X CGAA AGUCCCGU	2156	ACGGGACUC GAGUGAUG	2597
1406	AAUCUCCC CUGAUGAG X CGAA AUCAUCAC	2157	GUGAUGAUU GGGAGAUU	2598
1414	CCAUCAGG CUGAUGAG X CGAA AUCUCCCA	2158	UGGGAGAUU CCUGAUGG	2599
1415	CCCAUCAG CUGAUGAG X CGAA AAUCUCCC	2159	GGGAGAUUC CUGAUGGG	2600
1429	CCCACUGU CUGAUGAG X CGAA AUCUGCCC	2160	GGGAGAUU ACAGUGGG	2601
1430	UCCCCACUG CUGAUGAG X CGAA AAUCUGCC	2161	GGCAGAUUA CAGUGGGA	2602
1447	CCAGAUCC CUGAUGAG X CGAA AAUCUUUG	2162	CAAAGAAAUU GGAUCUGG	2603
1452	AUGAUCCA CUGAUGAG X CGAA AAUCAAAU	2163	AAUUGGAUC UGGAUCAU	2604
1458	UCCCCAAU CUGAUGAG X CGAA AUCCAGAU	2164	AUCUGGAUC AUUUGGAA	2605
1461	CUGUUCCA CUGAUGAG X CGAA AUGAUCCA	2165	UGGAUCAUU UGGAACAG	2606
1462	ACUGUUCC CUGAUGAG X CGAA AAUGAUCC	2166	GGAUCAJUU GGAACAGU	2607
1471	CCCUUGUA CUGAUGAG X CGAA ACUGUUCC	2167	GGAACAGUC UACAAGGG	2608
1473	UCCCCUUG CUGAUGAG X CGAA AGACUGUU	2168	AACAGUCUA CAAGGGAA	2609
1512	UCACAUUC CUGAUGAG X CGAA ACAUUUUC	2169	GAAAAAUGUU GAAUGUGA	2610
1529	CUGAGGUG CUGAUGAG X CGAA AGGUGCUG	2170	CAGCACCUA CACCUCAG	2611
1535	UAACUGCU CUGAUGAG X CGAA AGGUGUAG	2171	CUACACCUC AGCAGUUA	2612
1542	AGGCUUGU CUGAUGAG X CGAA ACUGCUGA	2172	UCAGCAGUU ACAAGCCU	2613
1543	AAGGCUUG CUGAUGAG X CGAA AACUGCUG	2173	CAGCAGUUU CAAGCCUU	2614
1551	CAUUUUUG CUGAUGAG X CGAA AGGUUUGU	2174	ACAAGCCUU CAAAAAUG	2615
1552	UCAUUUUU CUGAUGAG X CGAA AAGGCUU	2175	CAAGCCUUU AAAAUGA	2616
1564	AQUACUCC CUGAUGAG X CGAA ACUUCAU	2176	AAUGAAGUA GGAGUACU	2617
1570	UUCUGUG CUGAUGAG X CGAA ACUCCUAC	2177	GUAGGAGUA CUCAGGAA	2618
1573	GUUUUCCU CUGAUGAG X CGAA AGUACUCC	2178	GGAGUACUC AGGAAAAC	2619
1595	GAGUAGGA CUGAUGAG X CGAA AUUCACAU	2179	AUGUGAAUA UCCUACUC	2620
1597	AAGAGUAG CUGAUGAG X CGAA AUAAUCAC	2180	GUGAAUACU CUACUCUU	2621
1600	AUGAAGAG CUGAUGAG X CGAA AGGAUAUU	2181	AAUAUCCUA CUCUCAU	2622
1603	CCCAUGAA CUGAUGAG X CGAA AGUAGGAU	2182	AUCCUACUC UUCAUGGG	2623
1605	AGCCCCAUG CUGAUGAG X CGAA AGAGUAGG	2183	CCUACUCUU CAUGGGCU	2624
1606	UAGCCCCAU CUGAUGAG X CGAA AAGAGUAG	2184	CUACUCUUC AUGGGCUA	2625
1614	UUGUGGAA CUGAUGAG X CGAA AGCCCCAUG	2185	CAUGGGCUA UUCCACAA	2626

nt. Position	Ribozym	SEQ ID. N	Substrate	SEQ ID. No.
1616	CUUUGUGG CUGAUGAG X CGAA AUAGCCC	2186	UGGGCUAUU CCACAAAG	2627
1617	GUUUGUG CUGAUGAG X CGAA AAUAGCCC	2187	GGGCUAUUC CACAAAGC	2628
1637	GGUACAA CUGAUGAG X CGAA AGCCAGUU	2188	AACUGGCUA UUGUUACC	2629
1639	UGGGUAAC CUGAUGAG X CGAA AUAGCCAG	2189	CUGGCUAUU GUUACCCA	2630
1642	CACUGGGU CUGAUGAG X CGAA ACAAUAGC	2190	GCUAUUGUU ACCCAGUG	2631
1643	CCACUGGG CUGAUGAG X CGAA AACAAUAG	2191	CUAUUGUA CCCAGUGG	2632
1662	ACAAGCUG CUGAUGAG X CGAA AGCCCUCA	2192	UGAGGGCUC CAGCUUGU	2633
1668	GGUGAUAC CUGAUGAG X CGAA AGCUGGAG	2193	CUCCAGCUU GUAUCACC	2634
1671	GAUGGUGA CUGAUGAG X CGAA ACAAGCUG	2194	CAGCUUGUA UCACCAUC	2635
1673	GAGAUGGU CUGAUGAG X CGAA AUACAAGC	2195	GCUGUUAUC ACCAUCUC	2636
1679	GAU AUGGA CUGAUGAG X CGAA AUGGUGAU	2196	AUCACCAUC UCCAUUAUC	2637
1681	AUGUAUAG CUGAUGAG X CGAA AGAUGGUG	2197	CACCAUCUC CAUAUCAU	2638
1685	CUCAUAGA CUGAUGAG X CGAA AUGGAGAU	2198	AUCUCCAUU UCAUUGAG	2639
1687	GUCUAAU CUGAUGAG X CGAA AAUUGGAG	2199	CUCCAUUAUC AUUGAGAC	2640
1690	UUGGUCUC CUGAUGAG X CGAA AUGAU AUG	2200	CAUAUCAUU GAGACCAA	2641
1701	UCAUCUCA CUGAUGAG X CGAA AUUUGGUC	2201	GACCAAAUU UGAGAUGA	2642
1702	AUCAUCUC CUGAUGAG X CGAA AAUUUGGU	2202	ACCAAAUUU GAGAUGAU	2643
1711	AUAAGUUU CUGAUGAG X CGAA AUCAUCUC	2203	GAGAUGAUC AAACUUUAU	2644
1717	AUAUCUAU CUGAUGAG X CGAA AGUUUGAU	2204	AUCAACUUU AUAGAUAU	2645
1718	AAUAUCUA CUGAUGAG X CGAA AAGUUUGA	2205	UCAAACUUU UAGAUAUU	2646
1720	GCAAAUAC CUGAUGAG X CGAA AAUAGUUU	2206	AAACUUUAU UGAUUGC	2647
1724	UCGUGCAA CUGAUGAG X CGAA AUCUAAU	2207	UUAUAGAUU UUGCACGA	2648
1726	UGUCGUGC CUGAUGAG X CGAA AAUUCUAU	2208	AUAGAUAUU GCACGACA	2649
1754	GUGUAAGU CUGAUGAG X CGAA AUCCAUGC	2209	GCAUGGAUU ACUUCACAC	2650
1755	CGGUGUAAG CUGAUGAG X CGAA AAUCCAUG	2210	CAUGGAUUU CUUACACG	2651
1758	UGGCGUGU CUGAUGAG X CGAA AGUAAUCC	2211	GGAUUACUU ACACGCCA	2652
1759	UUGGCGUG CUGAUGAG X CGAA AAGUAAUC	2212	GAUUAUUU CACGCCAA	2653
1770	GGAUAGAUU CUGAUGAG X CGAA ACUUGGCG	2213	CGCCAAGUC AAUCAUCC	2654
1774	CUGUGGAU CUGAUGAG X CGAA AAUGACUU	2214	AAGUCAAUC AUCCACAG	2655
1777	UCUCUGUG CUGAUGAG X CGAA AUGAUUGA	2215	UCAAUCAUC CACAGAGA	2656
1789	UUACUCUU CUGAUGAG X CGAA AGGUCUCU	2216	AGAGACCUC AAGAGUAA	2657
1796	UAAUAAU CUGAUGAG X CGAA ACUCUUGA	2217	UCAAGAGUA AUAAUUAU	2658
1799	AAAUAAU CUGAUGAG X CGAA AAUACUCU	2218	AGAGUAAA AUAAUAAA	2659
1802	AAGAAAUA CUGAUGAG X CGAA AAUAAUAC	2219	GUAAUAAA UAAUUCUU	2660
1804	UGAAGAAA CUGAUGAG X CGAA AAUAAUAAU	2220	AAUAAUAAA UUUUUUCA	2661
1806	CAUGAAGA CUGAUGAG X CGAA AAUAAUAA	2221	AAUAAUAAA UCUUCAUG	2662
1807	UCAUGAAG CUGAUGAG X CGAA AAUAAUAAU	2222	AAUAAUAAA CUUCAUGA	2663
1808	UUCAUGAA CUGAUGAG X CGAA AAAUAAU	2223	AAUAAUAAA UUCAUGAA	2664
1810	UCUUCAUG CUGAUGAG X CGAA AGAAAUAU	2224	AAUAAUAAA CAUGAAGA	2665
1811	GUCUUCAU CUGAUGAG X CGAA AAGAAAUA	2225	AAUAAUAAA UUUUCUUC AUGAAGAC	2666
1822	UUUACUGU CUGAUGAG X CGAA AGGUUCUC	2226	GAAGACCUC ACAGUAAA	2667
1828	CCUAAUUU CUGAUGAG X CGAA ACUGUGAG	2227	CUCACAGUA AAAAUAGG	2668
1834	AAAUCACC CUGAUGAG X CGAA AAUAAUAC	2228	GUAAAAAAA GGUGAUUU	2669
1841	UAGACCAA CUGAUGAG X CGAA AUCACCUA	2229	UAGGUGAUU UGGGUCUA	2670
1842	CUAGACCA CUGAUGAG X CGAA AAUACCU	2230	AGGUGAUUU UGGGUAG	2671
1843	CCUAGACC CUGAUGAG X CGAA AAAUCACC	2231	GGUGAUUUU GGUCUAGC	2672
1847	UGUAGCUA CUGAUGAG X CGAA ACCAAAUA	2232	AAUAAUAAA UAGCUACA	2673
1849	ACUGUAGC CUGAUGAG X CGAA AGACCAA	2233	UUUGGUCUA GCUACAGU	2674
1853	UUUCACUG CUGAUGAG X CGAA AGCUAGAC	2234	GUCUAGCUA CAGUGAAA	2675
1863	UCCAUCGA CUGAUGAG X CGAA AAUUCACU	2235	AGUGAAAUC UCGAUGGA	2676
1865	ACUCCAUC CUGAUGAG X CGAA AGAUUUC	2236	UGAAAUCUC GAUGGAGU	2677
1878	ACUGAUGG CUGAUGAG X CGAA ACCCACUC	2237	GAGUGGGUC CCAUCAGU	2678
1883	UUCAACU CUGAUGAG X CGAA AUGGGACC	2238	GGUCCCCAU AGUUUGAA	2679
1887	ACUGUUCA CUGAUGAG X CGAA ACUGAUGG	2239	CCAUCAUU UGAACAGU	2680
1888	AACUGUUC CUGAUGAG X CGAA AACUGAUG	2240	CAUCAGUUU GAACAGUU	2681

nt. Position	Ribozyme	SEQ ID. No.	Substrate	SEQ ID. No.
1896	AUCCAGAC CUGAUGAG X CGAA ACUGUUCA	2241	UGAACAGUU GUCUGGAU	2682
1899	UGGAUCCA CUGAUGAG X CGAA ACAACUGU	2242	ACAGUUGUC UGGAUCCA	2683
1905	ACAAAAAUG CUGAUGAG X CGAA AUCCAGAC	2243	GUCUGGAUC CAUUUUGU	2684
1909	AUCCACAA CUGAUGAG X CGAA AUGGAUCC	2244	GGAUCCAUU UUGUGGAU	2685
1910	CAUCCACA CUGAUGAG X CGAA AAUGGAUC	2245	GAUCCAUU UGUGGAUG	2686
1911	CCAUCAC CUGAUGAG X CGAA AAAUGGAU	2246	AUCCAUUU GUGGAUGG	2687
1930	AUUCUGAU CUGAUGAG X CGAA ACUUCUGG	2247	CCAGAAGUC AUCAGAAU	2688
1933	UGCAUUCU CUGAUGAG X CGAA AUGACUUC	2248	GAAGUCAUC AGAAUGCA	2689
1946	UGGAUUUU CUGAUGAG X CGAA AUCUUGCA	2249	UGCAAGAU AAAAUCCA	2690
1952	GCUGUAUG CUGAUGAG X CGAA AUUUUUAU	2250	AUAAAAAUCAUACAGC	2691
1956	GAAAGCUG CUGAUGAG X CGAA AUGGAUUU	2251	AAAUCCAUA CAGCUUUC	2692
1962	CUGACUGA CUGAUGAG X CGAA AGCUGUAU	2252	AUACAGCUU UCAGUCAG	2693
1963	UCUGACUG CUGAUGAG X CGAA AAGCUGJA	2253	UACAGCUUU CAGUCAGA	2694
1964	AUCUGACU CUGAUGAG X CGAA AAAGCUGU	2254	ACAGCUUUC AGUCAGAU	2695
1968	AUACAUUCU CUGAUGAG X CGAA ACUGAAAG	2255	CUUUCAGUC AGAUGUAU	2696
1975	AAUGCAUA CUGAUGAG X CGAA ACAUCUGA	2256	UCAGAUGUA UAUGCAAU	2697
1977	CAAUAGCA CUGAUGAG X CGAA AUACAUU	2257	AGAUGUAUA UGCAUUIJG	2698
1983	CAAUCCCA CUGAUGAG X CGAA AUGCAUAU	2258	AUAUGCAUU UGGGAUUG	2699
1984	ACAAUCCC CUGAUGAG X CGAA AAUGCAUA	2259	UAUGCAUUU GGGAUJGU	2700
1990	UACAGAAC CUGAUGAG X CGAA AUCCCAAA	2260	UUUGGGAUU GUUCUGUA	2701
1993	UCAUACAG CUGAUGAG X CGAA ACAAUCCC	2261	GGGAUUGUU CUGUAUGA	2702
1994	UUCAUACA CUGAUGAG X CGAA AACAAUCC	2262	GGAUUGUUC UGUAUGAA	2703
1998	UCAAUUCA CUGAUGAG X CGAA ACAGAAC	2263	GUUUCUGUA UGAAUUGA	2704
2004	CAGUCAUC CUGAUGAG X CGAA AUUCAUAC	2264	GUUAUGAUU GAUGACUG	2705
2019	AAAUAAGGU CUGAUGAG X CGAA ACUGUCCA	2265	UGGACAGUU ACCUUAUU	2706
2020	GAUUUAAGG CUGAUGAG X CGAA AACUGUCC	2266	GGACAGUUA CCUUAUUC	2707
2024	GUUUGAAU CUGAUGAG X CGAA AGGUAAUC	2267	AGUUACCUU AUUCAAAC	2708
2025	UGUUUGAA CUGAUGAG X CGAA AAGGUAAAC	2268	GUUACCUUA UUCAAACA	2709
2027	GAUGUUUG CUGAUGAG X CGAA AAUAGGU	2269	UACCUUAUU CAAACAU	2710
2028	UGAUGUUU CUGAUGAG X CGAA AAUAAGGU	2270	ACCUUUAUC AAACAUCA	2711
2035	CUGUUGUU CUGAUGAG X CGAA AUGUUUGA	2271	UCAAACAU AACAACAG	2712
2053	AAAUAUU CUGAUGAG X CGAA AUCUGGU	2272	GACCAGAUA AUUUUUAU	2713
2056	ACCAUAAA CUGAUGAG X CGAA AUUAUCUG	2273	CAGAUAAA UUUUAGGU	2714
2057	CACCAUA CUGAUGAG X CGAA AAUUAUCU	2274	AGAUAAA UUAUGGUG	2715
2058	CCACCAUA CUGAUGAG X CGAA AAAUUUAUC	2275	GAUAUUUUU UAUGGUGG	2716
2059	CCCACCAU CUGAUGAG X CGAA AAAUUUAU	2276	AAUAUUUU AUGGUGGG	2717
2060	UCCCCACCA CUGAUGAG X CGAA AAAAUUA	2277	UAAAAUUUA UGGUGGGA	2718
2076	GAGACAGG CUGAUGAG X CGAA AUCCUCGU	2278	ACGAGGAUA CCUGUCUC	2719
2082	GAUCUGGA CUGAUGAG X CGAA ACAGGUAU	2279	AUACCUGUC UCCAGAUC	2720
2084	GAGAUCUG CUGAUGAG X CGAA AGACAGGU	2280	ACCUGUCUC CAGAUCUC	2721
2090	CUUACUGA CUGAUGAG X CGAA AUCUGGAG	2281	CUCCAGAUC UCAGUAAG	2722
2092	ACCUUACU CUGAUGAG X CGAA AGAUCUGG	2282	CCAGAUCUC AGUAAGGU	2723
2096	CCGUACCU CUGAUGAG X CGAA ACUGAGAU	2283	AUCUCAGUA AGGUACGG	2724
2101	UUACUCCG CUGAUGAG X CGAA ACCUUACU	2284	AGUAAGGUA CGGAGUAA	2725
2108	UGGACAGU CUGAUGAG X CGAA ACUCCGU	2285	UACGGAGUA ACUGUCCA	2726
2114	GGCUUUUG CUGAUGAG X CGAA ACAGUUAC	2286	GUACUGUC CAAAGCC	2727
2133	CUGCCAUU CUGAUGAG X CGAA AUCUCUUC	2287	GAAGAGAUU AAUGGCAG	2728
2134	UCUGCCAU CUGAUGAG X CGAA AAUCUCUU	2288	AAGAGAUUA AUGGCAGA	2729
2149	UUCUUUUU CUGAUGAG X CGAA AGGCACUC	2289	GAGUGCCUC AAAAGAA	2730
2176	UGGGGAAA CUGAUGAG X CGAA AGUGGU	2290	AGACCACUC UUUCCCCA	2731
2178	UUUGGGGA CUGAUGAG X CGAA AGAGUGGU	2291	ACCACUCUU UCCCCAAA	2732
2179	AAUUGGGG CUGAUGAG X CGAA AAGAGUGG	2292	CCACUCUUU CCCAAAU	2733
2180	AAUUUGGG CUGAUGAG X CGAA AAAGAGUG	2293	CACUCUUUC CCCAAAU	2734
2188	GAGGCGAG CUGAUGAG X CGAA AUUUGGG	2294	CCCCAAAUU CUCGCCUC	2735
2189	AGAGGCAGA CUGAUGAG X CGAA AAUUUGGG	2295	CCCCAAAUUC UCGCCUCU	2736

nt. Positi n	Rib zym	SEQ ID. N.	Substrate	SEQ ID. No.
2191	AUAGAGGC CUGAUGAG X CGAA AGAAUUUG	2296	CAAAUUCUC GCCUCUAU	2737
2196	GCUAAUA CUGAUGAG X CGAA AGGGGAGA	2297	UCUCGCCUC UAUUGAGC	2738
2198	CAGCUAA CUGAUGAG X CGAA AGAGGGGA	2298	UCGCCUCUA UUGAGCUG	2739
2200	AGCAGCUC CUGAUGAG X CGAA AUAGAGGC	2299	GCCUCUAUU GAGCUGCU	2740
2217	UUGGCAAU CUGAUGAG X CGAA AGCGGGCC	2300	GGCCCGCUC AUUGCCAA	2741
2220	UUUUUGGC CUGAUGAG X CGAA AUGAGCGG	2301	CCGCUCAUU GCCAAAAAA	2742
2230	CUGCGGUG CUGAUGAG X CGAA AUUUUUGG	2302	CAAAAUUUU CACCGCAG	2743
2231	ACUGCGGU CUGAUGAG X CGAA AUUUUUGG	2303	CAAAAUUC ACCGCAGU	2744
2244	AGGGUUCU CUGAUGAG X CGAA AUGCACUG	2304	CAGUGCauc AGAACCCU	2745
2253	GAUUAAG CUGAUGAG X CGAA AGGGUUCU	2305	AGAACCCUC CUUGAAUC	2746
2256	CCCGAUUC CUGAUGAG X CGAA AGGAGGGU	2306	ACCCUCCUU GAAUCGGG	2747
2261	ACCAGCCC CUGAUGAG X CGAA AUUCAAGG	2307	CCUUGAAUC GGGCUGGU	2748
2270	UGUUGGGA CUGAUGAG X CGAA ACCAGCCC	2308	GGGCUGGUU UCCAAACA	2749
2271	CUGUUUGG CUGAUGAG X CGAA AACCAAGC	2309	GGCUGGUUU CAAACAG	2750
2272	UCUGUUUG CUGAUGAG X CGAA AAACCAGC	2310	GCUGGUUUU CAAACAGA	2751
2285	UAGACUA CUGAUGAG X CGAA AUCCUCUG	2311	CAGAGGAUU UUAGUCUA	2752
2286	AUAGACUA CUGAUGAG X CGAA AAUCCUCU	2312	AGAGGAUUU UAGUCUAU	2753
2287	UAUAGACU CUGAUGAG X CGAA AAAUCCUC	2313	GAGGAUUUU AGUCUAUA	2754
2288	UAUAGAC CUGAUGAG X CGAA AAAAUCCU	2314	AGGAUUUUU GUCUAUUA	2755
2291	AGCAUUA CUGAUGAG X CGAA ACUAAA	2315	AUUUAGUC UAU AUGCU	2756
2293	CAAGCAUA CUGAUGAG X CGAA AGACUAAA	2316	UUUAGUCUA UAU AUGCUUG	2757
2295	CACAAGCA CUGAUGAG X CGAA AUAGACUA	2317	UAGUCUAUA UGCUUGUG	2758
2300	AGAAGCAC CUGAUGAG X CGAA AGCAUUA	2318	UAAAUGCUU GUGCUUCU	2759
2306	UUUUGGAG CUGAUGAG X CGAA AGCACAAG	2319	CUUGUGCUU CUCCAAAA	2760
2307	UUUUUGGA CUGAUGAG X CGAA AAGCACAA	2320	UUGUGCUUC UCCAAAAAA	2761
2309	UGUUUUUG CUGAUGAG X CGAA AGAACGAC	2321	GUGCUUCUC CAAAAACA	2762
2323	CCUGCCUG CUGAUGAG X CGAA AUGGGUGU	2322	ACACCAUC CAGGCAGG	2763
2337	ACGCACCA CUGAUGAG X CGAA AUCCCCCU	2323	AGGGGGUA UGGUGCGU	2764
2346	GGACAGGA CUGAUGAG X CGAA ACGCACCA	2324	UGGUGCGUU UCCUGUCC	2765
2347	UGGACAGG CUGAUGAG X CGAA AACGCACC	2325	GGUGCGUUU CCUGUCCA	2766
2348	GUGGACAG CUGAUGAG X CGAA AAACGCAC	2326	GUGCGUUUC CUGUCCAC	2767
2353	UUUCAGUG CUGAUGAG X CGAA ACAGGAAA	2327	UUUCCUGUC CACUGAAA	2768
2379	CUCUCCUG CUGAUGAG X CGAA ACUCUCU	2328	GAGAGAGUU CAGGAGAG	2769
2380	ACUCUCCU CUGAUGAG X CGAA AACUCUCU	2329	AGAGAGUUC AGGAGAGU	2770
2389	UUUGUUGC CUGAUGAG X CGAA ACUCUCCU	2330	AGGAGAGUA GCAACAAA	2771
2406	UGUUCAUU CUGAUGAG X CGAA AUUUUCCU	2331	AGGAAAUA AAUGAAC	2772
2416	AGCAAAAC CUGAUGAG X CGAA AUGUUCAU	2332	AUGAACAUU UGUUUGCU	2773
2420	UAUAAGCA CUGAUGAG X CGAA ACAUAUGU	2333	ACAUUAUGUU UGCUUAUA	2774
2421	UAUAUAAGC CUGAUGAG X CGAA AACAUUAUG	2334	CAUAUAUGUU GCUUAUAU	2775
2425	UAACAUAU CUGAUGAG X CGAA AGCAAAAC	2335	UGUUUUGCUU AU AUGUUA	2776
2426	UUAACAUUA CUGAUGAG X CGAA AAGCAAC	2336	GUUUUGCUUA UAUGUUA	2777
2428	AUUUAACA CUGAUGAG X CGAA AUAGCAA	2337	UGUCUUUAUA UGUUAAA	2778
2432	UUCAAUUU CUGAUGAG X CGAA ACAUAUAA	2338	UUUAUAUGUU AAAUUGAA	2779
2433	AUUCAAUU CUGAUGAG X CGAA AACAUUA	2339	UUAUAUGUU AAUUGAAU	2780
2437	UUUUAUUC CUGAUGAG X CGAA AUUUUACA	2340	UGUUAAAUA GAAUAAA	2781
2442	GAGUAUUU CUGAUGAG X CGAA AUUCAAUU	2341	AAUUGAAUA AAAUACUC	2782
2447	AAAGAGAG CUGAUGAG X CGAA AUUUUAUU	2342	AAUAAAUA CUCUCUUU	2783
2450	AAAAAAAGA CUGAUGAG X CGAA AGUAUUUU	2343	AAAAAAACUC UCUUUUUU	2784
2452	AAAAAAA CUGAUGAG X CGAA AGAGUAUU	2344	AAUACUCUC UUUUUUUU	2785
2454	AAAAAAA CUGAUGAG X CGAA AGAGAGUA	2345	UACUCUCUU UUUUUUUU	2786
2455	UUAAAAAA CUGAUGAG X CGAA AAGAGAGU	2346	ACUCUCUUU UUUUUUAA	2787
2456	UUAAAAAA CUGAUGAG X CGAA AAAGAGAG	2347	CUCUCUUUU UUUUUAG	2788
2457	CCUUAAAA CUGAUGAG X CGAA AAAAGAGA	2348	UCUCUUUUU UUUUAAGG	2789
2458	ACCUUAAA CUGAUGAG X CGAA AAAAAGAG	2349	CUCUUUUUU UUUAAAGG	2790
2459	CACCUUAA CUGAUGAG X CGAA AAAAAAGA	2350	UCUUUUUUU UUAAGGUG	2791

nt. Position	Ribozyme	SEQ ID. No.	Substrate	SEQ ID. N
2460	CCACCUUA CUGAUGAG X CGAA AAAAAAAG	2351	CUUUUUUUUUAAGGUGG	2792
2461	UCCACCUU CUGAUGAG X CGAA AAAAAAAA	2352	UUUUUUUUUUAAGGUGGA	2793
2462	UUCCACCU CUGAUGAG X CGAA AAAAAAAA	2353	UUUUUUUUUAAGGUGGAA	2794

Where "X" represents stem II region of a HH ribozyme (Hertel et al., 1992 Nucleic Acids Res. 20: 3252). The length of stem II may be  $\geq$  2 base-pairs.

Table XVII: Human B-raf Hairpin Ribozyme and Target Sequence

nt. Position	Ribozyme Sequence	SEQ ID. No.	Target Sequence	SEQ ID. No.
9	GGAGGG AGAA GCGA ACCAGAGAAACA X GUACAUUACCUUGUA	2795	UCCCG GCC CCCUCC	2846
20	CUGUCC AGAA GCGA ACCAGAGAAACA X GUACAUUACCUUGUA	2796	UCCCC GCC CGACAG	2847
31	CCGAGC AGAA GCUG ACCAGAGAAACA X GUACAUUACCUUGUA	2797	CAGCG GCC GCUCGG	2848
34	GGCCCC AGAA GCGG ACCAGAGAAACA X GUACAUUACCUUGUA	2798	CGGCC GCU CGGCC	2849
46	ACCGAG AGAA GGGG ACCAGAGAAACA X GUACAUUACCUUGUA	2799	CCCCG GCU CUCGGU	2850
114	CCGUUG AGAA GAGC ACCAGAGAAACA X GUACAUUACCUUGUA	2800	GCUCU GUU CAOOGG	2851
149	CGGCC AGAA GCGG ACCAGAGAAACA X GUACAUUACCUUGUA	2801	CGCCG GCC GCCCCG	2852
153	GCGCG AGAA GGGC ACCAGAGAAACA X GUACAUUACCUUGUA	2802	GGCCG GCC GCGGGC	2853
160	CGAAGA AGAA GCGG ACCAGAGAAACA X GUACAUUACCUUGUA	2803	CCGCG GCC UCUUCG	2854
169	GUCCGC AGAA GAAG ACCAGAGAAACA X GUACAUUACCUUGUA	2804	CUCGC GCU CGGGAC	2855
175	CCGAGG AGAA GCGAG ACCAGAGAAACA X GUACAUUACCUUGUA	2805	CUGCG GAC CGUGCC	2856
379	AGAAAA AGAA GUUC ACCAGAGAAACA X GUACAUUACCUUGUA	2806	GAACU GAU UUUUCU	2857
388	GCUAGA AGAA GAAG ACCAGAGAAACA X GUACAUUACCUUGUA	2807	UUUCU GUU UCUAGC	2858
466	UUGAAA AGAA GAAG ACCAGAGAAACA X GUACAUUACCUUGUA	2808	UUUCA GUU UUUCAA	2859
484	UGCCAC AGAA GUUG ACCAGAGAAACA X GUACAUUACCUUGUA	2809	CCACA GAU GUCCCA	2860
540	UUGUUG AGAA GGAA ACCAGAGAAACA X GUACAUUACCUUGUA	2810	UUCUU GCC CAACAA	2861
586	GUUCUCG AGAA GUUA ACCAGAGAAACA X GUACAUUACCUUGUA	2811	UUACA GUC CGAGAC	2862
596	UCUUUA AGAA GUUC ACCAGAGAAACA X GUACAUUACCUUGUA	2812	AGACA GUC UAAAGA	2863
612	CUCAUU AGAA GUGC ACCAGAGAAACA X GUACAUUACCUUGUA	2813	GCACU GAU GAUGAG	2864
646	UCDUGA AGAA GCAC ACCAGAGAAACA X GUACAUUACCUUGUA	2814	GUGCU GUU UACAGA	2865
819	UGGAAA AGAA GUUU ACCAGAGAAACA X GUACAUUACCUUGUA	2815	AAGCU GCU UUUCCA	2866
836	UUUGAC AGAA GAAG ACCAGAGAAACA X GUACAUUACCUUGUA	2816	UUUCC GCU GUCAA	2867
891	ACACAC AGAA GUGG ACCAGAGAAACA X GUACAUUACCUUGUA	2817	CCACU GAU GUUGGU	2868
924	GAGACA AGAA GCGA ACCAGAGAAACA X GUACAUUACCUUGUA	2818	UUGCU GUU UGUCUC	2869
988	UGUUAG AGAA GUUC ACCAGAGAAACA X GUACAUUACCUUGUA	2819	AGACU GCC CUAACA	2870
1021	GUCCGA AGAA GGUG ACCAGAGAAACA X GUACAUUACCUUGUA	2820	CACCC GCC UCGGAC	2871
1027	AAUAGA AGAA GAGC ACCAGAGAAACA X GUACAUUACCUUGUA	2821	CCUCG GAC UCUAUU	2872
1055	GAGACG AGAA GGUG ACCAGAGAAACA X GUACAUUACCUUGUA	2822	CACCA GUC CGUCUC	2873
1059	GAAGGA AGAA GCU ACCAGAGAAACA X GUACAUUACCUUGUA	2823	AGUCC GUC UCUUUC	2874
1089	CGGAAG AGAA GUUG ACCAGAGAAACA X GUACAUUACCUUGUA	2824	CCACA GCC CUUCCG	2875
1097	CUGCUU AGAA GAGC ACCAGAGAAACA X GUACAUUACCUUGUA	2825	CUUCC GAC CAGCAG	2876
1105	AUCUUUC AGAA GCGU ACCAGAGAAACA X GUACAUUACCUUGUA	2826	CAGCA GAU GAAGAU	2877
1142	AUGAGG AGAA GCUU ACCAGAGAAACA X GUACAUUACCUUGUA	2827	AGACCG GAU CCUCAU	2878
1153	AUUGGG AGAA GAUG ACCAGAGAAACA X GUACAUUACCUUGUA	2828	CAUCA GCU CCCAAU	2879
1267	UAUAUG AGAA GGGG ACCAGAGAAACA X GUACAUUACCUUGUA	2829	CCCCU GCU UCAUUA	2880
1417	CUGCCC AGAA GGAA ACCAGAGAAACA X GUACAUUACCUUGUA	2830	UUCU GAU GGGCAG	2881
1425	ACUGUA AGAA GCCC ACCAGAGAAACA X GUACAUUACCUUGUA	2831	GGCA GAU UACAGU	2882
1468	CUUGUA AGAA GUUC ACCAGAGAAACA X GUACAUUACCUUGUA	2832	GAACA GUC UACAAG	2883

nt. Position	Ribozyme Sequence	SEQ ID. No.	Target Sequence	SEQ ID. No.
1664	GAUACA AGAA GGAG ACCAGAGAAAC X GUACAUUACCUGGUA	2833	CUCCA GCU UGUUAUC	2884
1734	UGUICCA AGAA GUCC ACCAGAGAAAC X GUACAUUACCUGGUA	2834	CGACA GAC UGCACA	2885
1884	UGUUCA AGAA GAUG ACCAGAGAAAC X GUACAUUACCUGGUA	2835	CAUCA GUU UGAACCA	2886
1893	CCAGAC AGAA GUUC ACCAGAGAAAC X GUACAUUACCUGGUA	2836	GAACA GUU GUCCUGG	2887
1958	ACUGGA AGAA GUAU ACCAGAGAAAC X GUACAUUACCUGGUA	2837	AUACA GCU UUCAGU	2888
1969	AUAUAC AGAA GACU ACCAGAGAAAC X GUACAUUACCUGGUA	2838	AGUCA GAU GUAUAU	2889
1995	AAUUCA AGAA GAAC ACCAGAGAAAC X GUACAUUACCUGGUA	2839	GUUCU GUA UGAAAU	2890
2079	UCUGGA AGAA GGUA ACCAGAGAAAC X GUACAUUACCUGGUA	2840	UACCU GUC UCCAGA	2891
2086	ACUGAG AGAA GGAG ACCAGAGAAAC X GUACAUUACCUGGUA	2841	CUCCA GAU CUAGU	2892
2111	CUUUG AGAA GUUA ACCAGAGAAAC X GUACAUUACCUGGUA	2842	UUAUCU GUC CAAAG	2893
2205	CGGGCC AGAA GCUC ACCAGAGAAAC X GUACAUUACCUGGUA	2843	GAGCU GCU GGCCCG	2894
2213	GCAAG AGAA GGCC ACCAGAGAAAC X GUACAUUACCUGGUA	2844	GGGCC GCU CAUUGC	2895
2350	UCAGUG AGAA GGAA ACCAGAGAAAC X GUACAUUACCUGGUA	2845	UUCCU GUC CACUGA	2896

Where "X" represents stem IV region of a Hairpin ribozyme. The length of stem IV may be  $\geq 2$  base-pairs.

**Table XVIII. Hammerhead (HH) Ribozyme target with sequence homology between c-raf and A-raf**

nt. Position	Target	Seq I.D. No.
452	AGGAGCU C AUUGUCG	2897
527	UGGCGUU C UGUGACU	2898
583	UGUGGCU A CAAGUUC	2899
668	ACAGUGU C CAGGAUU	2900
857	AUAUGGU C AGCACCA	2901
1096	UCAGGCU A UUACUGG	2902
1098	AGGCUAU U ACUGGGA	2903
1246	CAGGCUU U CAAGAAU	2904
1247	AGGCUUU C AAGAAUG	2905
1309	AUGGGCU U CAUGACC	2906
1327	CCGGGAU U UGCCAUC	2907
1357	GAGGGCU C CAGCCUC	2908
1412	UCCAGCU C AUCGACG	2909
1469	AGAACAU C AUCCACC	2910
1628	AGGUGAU C CGUAUGC	2911
1658	ACAGCUU C CAGUCAG	2912
1663	UUCCAGU C AGACGUC	2913
1748	ACCAGAU U AUCUUUA	2914
1749	CCAGAUU A UCUUUUAU	2915
1751	AGAAUUAU C UUUUAUGG	2916
1753	AUUAUCU U UAUGGUG	2917
1754	UUAAUCUU U AUGGUGG	2918
1871	GGCCCCU C UUCCCCC	2919
1874	CCCUUU C CCCCAGA	2920
1951	CCCUCCU U GCACCGC	2921
2046	CCAAUCU C AGCCCUC	2922
2127	CCCCAUU C CCCACCC	2923
2174	AGUUCUU C UGGAAUU	2924
2251	UGGGGAU A CUUCUAA	2925
2400	GUCCUU U UGUGUCU	2926
2432	CUCCUCU C UUUCUUC	2927

**Table XIX. Hammerhead Ribozyme Target with sequence homology between c-raf and B-raf**

nt. Position	Target Sequence	Seq. I. D. No.
17	GCCCCU C CCCGCC	2928
405	UCUGCAU C AAUGGAU	2929
426	ACAUUU C UUCCUCU	2930
479	UCAAAAU C CCACAGA	2931
702	GAUAUUU C CUGGCUU	2932
754	UCCACU U ACAACAC	2933
861	UAUAAA U UCACCAG	2934
931	UGUUUGU C UCCAAGU	2935
1034	GGACUCU A UUGGGCC	2936
1259	GUCUGCU A CCCCCCC	2937
1344	AAGUCAU C UUCAUCC	2938
1603	UCCUACU C UUCAUGG	2939
1662	GAGGGCU C CAGCUUG	2940
1802	UAUAAA A UAUUUCU	2941
1804	AUAUAU A UUUCUUC	2942
1806	AAUAAU U UCUUCAU	2943
1807	AUAUAU U CUUCAUG	2944
1808	UAUAAA U UUCAUGA	2945
1810	UAUUUCU U CAUGAAG	2946
1834	AAAAAAU A GGUGAUU	2947
1842	GGUGAUU U UGGUCUA	2948
1847	UUUUGGU C UAGCUAC	2949
1956	AAUCCAU A CAGCUUU	2950
2035	CAAACAU C AACAAACA	2951
2059	UAUUUU U AUGGUGG	2952
2090	UCCAGAU C UCAGUAA	2953
2092	CAGAUCU C AGUAAGG	2954
2200	CCUCUAU U GAGCUGC	2955
2256	CCCUCCU U GAAUCGG	2956

Table XX.

<b>Experimental Group</b>	<b>Ribozyme Activity/Target</b>	<b>Dose (mg/kg/day)</b>	<b>Sample Size per dose</b>
RPI.4610	Active/ <i>flt-1</i>	1, 3, 10, 30, 100	10
RPI.4611	Inactive/ <i>flt-1</i>	1, 3, 10, 30, 100	10
RPI.4733	Active/ <i>flk-1</i>	1, 3, 10, 30, 100	10
RPI.4734	Inactive/ <i>flk-1</i>	1, 3, 10, 30, 100	10
Saline	NA	12 $\mu$ l/day	10

Claims

1. A method for identification of a nucleic acid molecule capable of modulating a process in a biological system comprising the steps of:
  - a) introducing a random library of a nucleic acid catalyst with a substrate binding domain and a catalytic domain, wherein said substrate binding domain comprises a random sequence, into said biological system under conditions suitable for modulating said process; and
  - b) determining the nucleotide sequence of at least a portion of the substrate binding domain of said nucleic acid catalyst from a said biological system in which the process has been modulated.
2. A method for identifying one or more nucleic acid molecules involved in a process in a biological system comprising the steps of:
  - a) providing a library of a nucleic acid catalyst, with a substrate binding domain and a catalytic domain, wherein said substrate binding domain comprises a random sequence, to said biological system under conditions suitable for said process to be altered;
  - b) identifying any said nucleic acid catalyst present in said biological system where said process has been altered by said any said nucleic acid catalyst; and
  - c) determining the nucleotide sequence of at least a portion of the binding arm of said any said nucleic acid catalyst to allow said identification of said nucleic acid molecule involved in said process in said biological system.
3. A method for identification of a nucleic acid catalyst capable of modulating a process in a biological system comprising the steps of:
  - a) introducing a random library of a nucleic acid catalyst with a substrate binding domain and a catalytic domain, wherein said substrate binding domain comprises

a random sequence, into said biological system under conditions suitable for modulating said process; and

b) identifying said nucleic acid catalyst from said biological system in which the process has been modulated.

5        4. The method of any of claims 1-3, wherein said biological system is a bacterial cell.

5        5. The method of any of claims 1-3, wherein said biological system is of plant origin.

10        6. The method of any of claims 1-3, wherein said biological system is of mammalian origin.

7. The method of any of claims 1-3, wherein said biological system is of yeast origin.

8. The method of any of claims 1-3, wherein said biological system is *Drosophila*.

9. The method of any of claims 1-3, wherein said nucleic acid catalyst is in a hammerhead motif.

15        10. The method of any of claims 1-3, wherein said nucleic acid catalyst is in a hairpin motif.

11. The method of any of claims 1-3, wherein said nucleic acid catalyst is in a hepatitis delta virus ribozyme motif.

20        12. The method of any of claims 1-3, wherein said nucleic acid catalyst is in group I intron, group II intron, VS ribozyme or RNase P ribozyme motif.

13. The method of any of claims 1-3, wherein said process is selected from the group consisting of growth, proliferation, apoptosis, morphology, angiogenesis, differentiation, migration, viral multiplication, drug resistance, signal

transduction, cell cycle regulation, temperature sensitivity and chemical sensitivity.

14. The method of any of claims 1-3, wherein said random library of nucleic acid catalysts is encoded by an expression vector in a manner which allows expression of said nucleic acid catalysts.  
5
15. The method of claim 14, wherein said expression vector comprises:
  - a) a transcription initiation region;
  - b) a transcription termination region;
  - c) a gene encoding at least one said nucleic acid catalyst; and

10 wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid catalyst.

- 16. The method of claim 14, wherein said expression vector comprises:
  - a) a transcription initiation region;
  - b) a transcription termination region;
  - 15 c) an open reading frame;
  - d) a gene encoding at least one said nucleic acid catalyst, wherein said gene is operably linked to the 3'-end of said open reading frame; and

20 wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid catalyst.

- 17. The method of claim 14, wherein said expression vector comprises:

- a) a transcription initiation region;
- b) a transcription termination region;
- c) an intron;
- d) a gene encoding at least one said nucleic acid catalyst; and

5       wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid catalyst.

18. The method of claim 14, wherein said expression vector comprises:

- a) a transcription initiation region;
- b) a transcription termination region;
- c) an intron;
- d) an open reading frame;
- e) a gene encoding at least one said nucleic acid catalyst, wherein said gene is operably linked to the 3'-end of said open reading frame; and

15       wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid catalyst.

19. The method of claim 14, wherein said expression vector is derived from a retrovirus.

20. The method of claim 14, wherein said expression vector is derived from an adenovirus.

21. The method of claim 14, wherein said expression vector is derived from an adeno-associated virus.
22. The method of claim 14, wherein said expression vector is derived from an alphavirus.
- 5 23. The method of claim 14, wherein said expression vector is derived from a bacterial plasmid.
24. The method of claim 14, wherein said expression vector is operable linked to a RNA polymerase II promoter element.
25. The method of claim 14, wherein said expression vector is operable linked to a 10 RNA polymerase III promoter element.
26. The method of claim 25, wherein said RNA polymerase III promoter is derived from a transfer RNA gene.
27. The method of claim 25, wherein said RNA polymerase III promoter is derived from a U6 small nuclear RNA gene.
- 15 28. The method of claim 25, wherein the nucleic acid catalyst comprises a sequence at its 5'-end homologous to the terminal 27 nucleotides of encoded by said U6 small nuclear RNA gene.
29. The method of claim 28, wherein said RNA polymerase III promoter is derived from a TRZ RNA gene.
- 20 30. The method of any of claims 1-3, wherein said biological system is of an eukaryotic origin.
31. The method of any of claims 1-3, wherein said biological system is of an prokaryotic origin.

5

32. The method of any of claims 1-3, wherein said biological system is of an archaebacterial origin.

33. The method of any of claims 1-3, wherein said substrate binding domain of the nucleic acid catalyst is of length sufficient to form a stable interaction with a target sequence.

34. The method of claim 33, wherein said substrate binding domain is of length between 12 and 100 nucleotides.

35. The method of claim 33, wherein said substrate binding domain is of length between 14 and 24 nucleotides.

10

36. The method of any of claims 1-3, wherein said nucleic acid catalyst comprises one substrate binding arm.

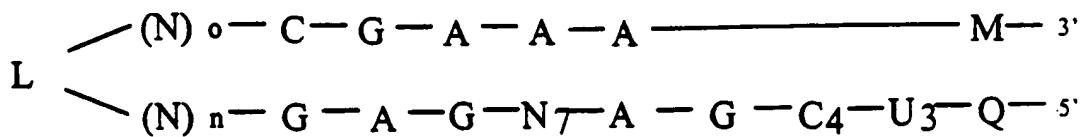
37. The method of any of claims 1-3, wherein said nucleic acid catalyst comprises two substrate binding arms.

15

38. The method of claim 37, wherein said substrate binding arms are of similar length.

39. The method of claim 37, wherein said substrate binding arms are of different length.

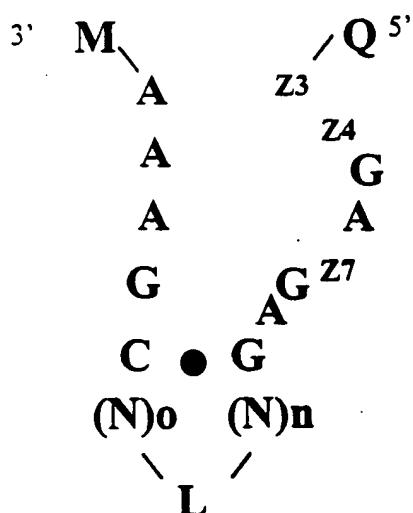
40. A nucleic acid molecule with an endonuclease activity having the formula III:



20 wherein, N is independently a nucleotide or a non-nucleotide linker, which may be same or different; M and Q are independently oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; o and n are

integers greater than or equal to 1, wherein if  $(N)_o$  and  $(N)_n$  are nucleotides,  $(N)_o$  and  $(N)_n$  are optionally able to interact by hydrogen bond interaction; L is a linker which may be present or absent, but when present, is a nucleotide and/or a non-nucleotide linker, which may be a single-stranded and/or double-stranded region; \_\_\_\_\_ represents a chemical linkage; and A, C, U and G represent adenosine, cytidine, uridine and guanosine nucleotides, respectively.

5 41. A nucleic acid molecule with catalytic activity having the formula IV:

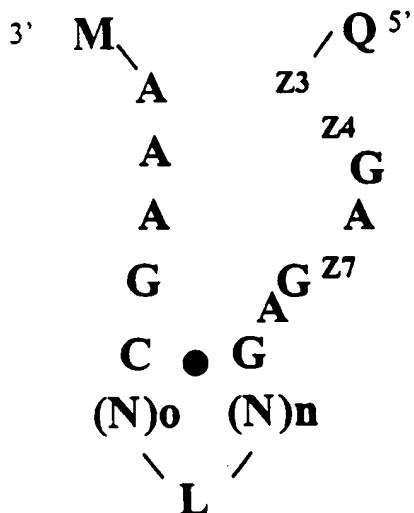


10 wherein, N is independently a nucleotide or a non-nucleotide linker, which may be same or different; M and Q are independently oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; o and n are integers greater than or equal to 1, wherein if  $(N)_o$  and  $(N)_n$  are nucleotides,  $(N)_o$  and  $(N)_n$  are optionally able to interact by hydrogen bond interaction; L is a linker which may be present or absent, but when present, is a nucleotide and/or a non-nucleotide linker, which may be a single-stranded and/or double-stranded region; Z3 is 2'-methylthiomethyl uridine; Z4 is 2'-C-allyl uridine; Z7 is 6-methyl uridine; \_\_\_\_\_ represents a chemical linkage; and A, and G represent adenosine and guanosine nucleotides, respectively.

15

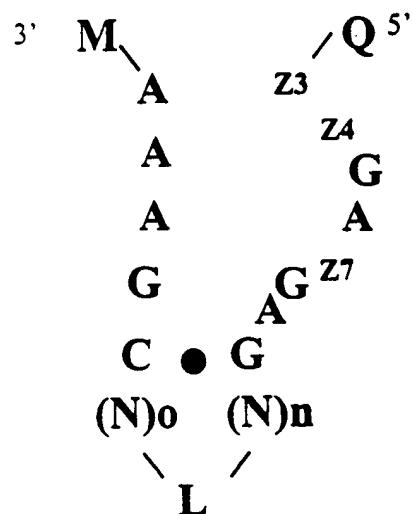
42. A nucleic acid molecule with catalytic activity having the formula

V:



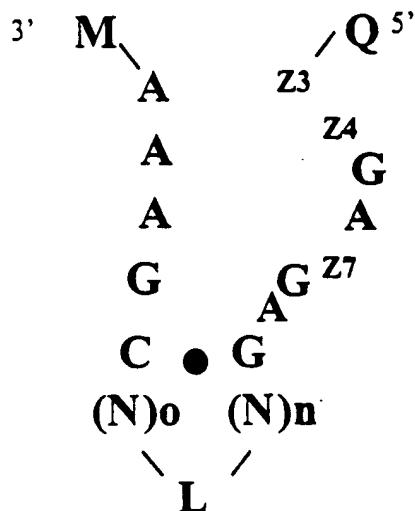
wherein, N is independently a nucleotide or a non-nucleotide linker, which may be same or different; M and Q are independently oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; o and n are integers greater than or equal to 1, wherein if (N)<sub>o</sub> and (N)<sub>n</sub> are nucleotides, (N)<sub>o</sub> and (N)<sub>n</sub> are optionally able to interact by hydrogen bond interaction; L is a linker which may be present or absent, but when present, is a nucleotide and/or a non-nucleotide linker, which may be a single-stranded and/or double-stranded region; Z3 is 2'-methylthiomethyl uridine; Z4 is 2'-methylthiomethyl cytidine; Z7 is 6-methyl uridine; \_\_\_\_\_ represents a chemical linkage; and A, and G represent adenosine and guanosine nucleotides, respectively.

43. A nucleic acid molecule with catalytic activity having the formula VI:



5       wherein, N is independently a nucleotide or a non-nucleotide linker, which may  
 be same or different; M and Q are independently oligonucleotides of length  
 sufficient to stably interact with a target nucleic acid molecule; o and n are  
 integers greater than or equal to 1, wherein if (N)o and (N)n are nucleotides, (N)o  
 and (N)n are optionally able to interact by hydrogen bond interaction; L is a  
 10      linker which may be present or absent, but when present, is a nucleotide and/or a  
 non-nucleotide linker, which may be a single-stranded and/or double-stranded  
 region; Z3 is 2'-methylthiomethyl uridine; Z4 is 2'-methylthiomethyl cytidine;  
 Z7 is 2'-C-allyl uridine; \_\_\_\_\_ represents a chemical linkage; and A, and G  
 represent adenosine and guanosine nucleotides, respectively.

44. A nucleic acid molecule with catalytic activity having the formula VII:



wherein, N is independently a nucleotide or a non-nucleotide linker, which may be same or different; M and Q are independently oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; o and n are integers greater than or equal to 1, wherein if (N)o and (N)n are nucleotides, (N)o and (N)n are optionally able to interact by hydrogen bond interaction; L is a linker which may be present or absent, but when present, is a nucleotide and/or a non-nucleotide linker, which may be a single-stranded and/or double-stranded region; Z3 is 2'-methylthiomethyl uridine; Z4 is 2'-methylthiomethyl cytidine; Z7 is pyridine-4-one; and        represents a chemical linkage; and A, and G represent adenosine and guanosine nucleotides, respectively.

45. The nucleic acid molecules of any of claims 40-44, wherein said (N)o and (N)n are nucleotides and said o and n are integers greater than or equal to 3.

46. The nucleic acid molecules of any of claims 40-44, wherein said L is nucleotide linker.

47. The nucleic acid molecule of any of claims 40-44, wherein said nucleic acid cleaves a separate nucleic acid molecule.

48. The nucleic acid molecule of claim 47, wherein said separate nucleic acid molecule is RNA.
49. The nucleic acid molecule of claim 47, wherein said nucleic acid comprises between 12 and 100 bases complementary to said separate nucleic acid molecule.
50. The nucleic acid molecule of claim 47, wherein said nucleic acid comprises between 14 and 24 bases complementary to said separate nucleic acid molecule.
51. A cell including the nucleic acid molecule of any of claims 40-44.
52. The cell of claim 17, wherein said cell is a mammalian cell.
53. The cell of claim 18, wherein said cell is a human cell.
- 10 54. An expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecule of any of claims 40-44, in a manner which allows expression of that nucleic acid molecule.
55. A cell including the expression vector of claim 54.
56. The cell of claim 55, wherein said cell is a mammalian cell.
- 15 57. The cell of claim 55, wherein said cell is a human cell.
58. A pharmaceutical composition comprising the nucleic acid molecule of any of claims 40-44.
59. A method for modulating expression of a gene in a plant cell by administering to said cell the nucleic acid molecule of any of claims 40-44.
- 20 60. A method for modulating expression of gene in a mammalian cell by administering to said cell the nucleic acid molecule of any of claims 40-44.

61. A method of cleaving a separate nucleic acid comprising, contacting the nucleic acid molecule of any of claims 40-44 with said separate nucleic acid molecule under conditions suitable for the cleavage of said separate nucleic acid molecule.
62. The method of claim 61, wherein said cleavage is carried out in the presence of a 5 divalent cation.
63. The method of claim 62, wherein said divalent cation is  $Mg^{2+}$ .
64. The nucleic acid molecule of claims 40-44, wherein said nucleic acid is chemically synthesized.
65. The expression vector of claim 54, wherein said vector comprises:
  - 10 a) a transcription initiation region;
  - b) a transcription termination region;
  - c) a gene encoding at least one said nucleic acid molecule; and

wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid 15 molecule.
66. The expression vector of claim 54, wherein said vector comprises:
  - a) a transcription initiation region;
  - b) a transcription termination region;
  - c) an open reading frame;
  - 20 d) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and

wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

67. The expression vector of claim 59, wherein said vector comprises:

- 5 a) a transcription initiation region;
- b) a transcription termination region;
- c) an intron;
- d) a gene encoding at least one said nucleic acid molecule; and

wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

10 68. The expression vector of claim 59, wherein said vector comprises:

- a) a transcription initiation region;
- b) a transcription termination region;
- 15 c) an intron;
- d) an open reading frame;
- e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and

20 wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

69. A method for identifying variants of a nucleic acid catalyst comprising the steps of:

5           a) selecting at least three positions within said nucleic acid catalyst to be varied with a predetermined group of different nucleotides;

10           b) synthesizing a first class of different pools of said nucleic acid catalyst, wherein the number of pools synthesized is equal to the number of nucleotides in the predetermined group of different nucleotides, wherein at least one of the positions to be varied in each pool comprises a defined nucleotide selected from the predetermined group of different nucleotides and the remaining positions to be varied comprise a random mixture of nucleotides selected from the predetermined group of different nucleotides;

15           c) testing the different pools of said nucleic acid catalyst under conditions suitable for said pools to show a desired attribute and identifying the pool with said desired attribute and wherein the position with the defined nucleotide in the pool with the desired attribute is made constant in subsequent steps;

20           d) synthesizing a second class of different pools of nucleic acid catalyst, wherein at least one of the positions to be varied in each of the second class of different pools comprises a defined nucleotide selected from the predetermined group of different nucleotides and the remaining positions to be varied comprise a random mixture of nucleotides selected from the predetermined group of different nucleotides;

25           e) testing the second class of different pools of said nucleic acid catalyst under conditions suitable for showing desired attribute and identifying the pool with said desired attribute and wherein the position with the defined nucleotide in the pool with the desired attribute is made constant in subsequent steps; and

          f) repeating the process similar to steps d and e until every position selected in said nucleic acid catalyst to be varied is made constant.

70. A method for identifying novel nucleic acid molecules in a biological system, comprising the steps of:

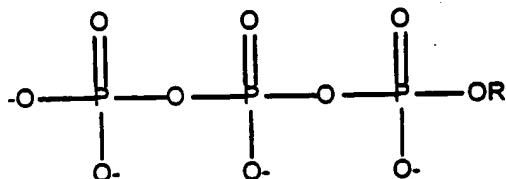
5                   a) synthesizing a pool of nucleic acid catalyst with a substrate binding domain and a catalytic domain, wherein said substrate binding domain comprises a random sequence;

10                  b) testing the pools of nucleic acid catalyst under conditions suitable for showing a desired effect in said biological system and identifying the catalyst showing said desired effect;

15                  c) using an oligonucleotide, comprising the sequence of the substrate binding domain of the nucleic acid catalyst showing said desired activity, as a probe, screening said biological system for nucleic acid molecules complementary to said probe; and

                        d) isolating and sequencing said complementary nucleic acid molecules.

71. A compound having the formula I:



wherein R is independently any nucleoside selected from the group consisting of 2'-O-methyl-2,6-diaminopurine riboside; 2'-deoxy-2' amino-2,6-diaminopurine riboside; 2'-(N-alanyl) amino-2'-deoxy-uridine; 2'-(N-phenylalanyl) amino-2'-deoxy-uridine; 2'-deoxy-2'-(N-β-alanyl) amino; 2'-deoxy-2'-(lysyl) amino uridine; 2'-C-allyl uridine; 2'-O-amino-uridine; 2'-O-methylthiomethyl adenosine; 2'-O-methylthiomethyl cytidine; 2'-O-methylthiomethyl guanosine; 2'-O-methylthiomethyl-uridine; 2'-Deoxy-2'-(N-histidyl) amino uridine; 2'-deoxy-2'-amino-5-methyl cytidine; 2'-(N-β-carboxamidine-β-alanyl) amino-2'-deoxy-uridine; 2'-deoxy-2'-(N-β-alanyl)-guanosine; and 2'-O-amino-adenosine.

72. A process for incorporation of the compounds of claim 71 into an oligonucleotide comprising the step of contacting said compound with a mixture comprising a nucleic acid template, an RNA polymerase enzyme, and an enhancer of modified nucleotide triphosphate incorporation, under conditions suitable for the incorporation of said compound into said oligonucleotide.  
5
73. The process of claim 72, wherein said RNA polymerase is a T7 RNA polymerase.
74. The process of claim 72, wherein said RNA polymerase is a mutant T7 RNA polymerase.
- 10 75. The process of claim 72, wherein said RNA polymerase is a SP6 RNA polymerase.
76. The process of claim 72, wherein said RNA polymerase is a mutant SP6 RNA polymerase.
- 15 77. The process of claim 72, wherein said RNA polymerase is a T3 RNA polymerase.
78. The process of claim 72, wherein said RNA polymerase is a mutant T3 RNA polymerase.
79. The process of claim 72, wherein said enhancer of modified nucleotide triphosphate incorporation is selected from the group consisting of LiCl, methanol, polyethylene glycol, diethyl ether, propanol, methylamine, and ethanol.  
20
80. A process for the synthesis of a pyrimidine nucleotide triphosphate comprising the steps of:
  - (a) monophosphorylation, wherein a pyrimidine nucleoside is contacted with a mixture comprising a phosphorylating reagent, a trialkyl phosphate and  
25

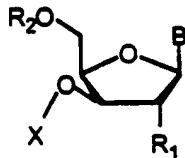
dimethylaminopyridine, under conditions suitable for the formation of a pyrimidine nucleotide monophosphate; and

(b) pyrophosphorylation, wherein said pyrimidine monophosphate from step (a) is contacted with a pyrophosphorylating reagent under conditions suitable for the formation of said pyrimidine nucleoside triphosphate.

5

81. The process of claim 80, wherein said pyrimidine nucleoside triphosphate is uridine triphosphate.
82. The process of claim 80, wherein said uridine triphosphate has a 2'-sugar modification.
- 10 83. The process of claim 82, wherein said uridine triphosphate is 2'-O-methylthiomethyl uridine triphosphate.
84. The process of claim 80, wherein said phosphorylating agent is selected from the group consisting of phosphorus oxychloride, phospho-tris-triazolides and phospho-tris-triimidazolides.
- 15 85. A process of claim 80, wherein said trialkylphosphate is triethyl phosphate.
86. The process of claim 80, wherein said pyrophosphorylating reagent is tributyl ammonium pyrophosphate.
87. The process of claim 72, wherein said oligonucleotide is RNA.
88. The process of claim 72, wherein said oligonucleotide is an nucleic acid catalyst.
- 20 89. The process of claim 72, wherein said oligonucleotide is an aptamer.
90. A kit for synthesis of an oligonucleotide comprising an RNA polymerase, an enhancer of modified nucleotide triphosphate incorporation and at least one compound of claim 71.

91. A kit for synthesis of an oligonucleotide comprising a DNA polymerase, an enhancer of modified nucleotide triphosphate incorporation and at least one compound of claim 71.
92. The kit of claim 90, wherein said RNA polymerase is a bacteriophage T7 RNA polymerase.  
5
93. The kit of claim 90, wherein said RNA polymerase is a bacteriophage SP6 RNA polymerase.
94. The kit of claim 90, wherein said RNA polymerase is a bacteriophage T3 RNA polymerase.
- 10 95. The kit of claim 90, wherein said RNA polymerase is a mutant T7 RNA polymerase.
96. The kit of claim 90 or 91, wherein said kit comprises at least two compounds of claim 71.
97. A compound having the formula II:



15

20

wherein, R<sub>1</sub> is OH, O-R<sub>3</sub>, wherein R<sub>3</sub> is independently a moiety selected from a group consisting of alkyl, alkenyl, alkynyl, aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester, C-R<sub>4</sub>, wherein R<sub>4</sub> is independently a moiety selected from a group consisting of alkyl, alkenyl, alkynyl, aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester, halo, NHR<sub>4</sub>, wherein R<sub>4</sub> is independently a moiety selected from a group consisting of alkyl (C1-22), acyl (C1-22), substituted or unsubstituted aryl), or OCH<sub>2</sub>SCH<sub>3</sub> (methylthiomethyl), ONHR<sub>5</sub>, where R<sub>5</sub> is independently a moiety selected from a group consisting of H, aminoacyl group, peptidyl group, biotinyl group, cholesteryl group, lipoic

acid residue, retinoic acid residue, folic acid residue, ascorbic acid residue, nicotinic acid residue, 6-aminopenicillanic acid residue, 7-aminocephalosporanic acid residue, alkyl, alkenyl, alkynyl, aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide or ester, ON=R<sub>6</sub>, where R<sub>6</sub> is independently pyridoxal residue, pyridoxal-5-phosphate residue, 13-cis-retinal residue, 9-cis-retinal residue, alkyl, alkenyl, alkynyl, alkylaryl, carbocyclic alkylaryl, or heterocyclic alkylaryl;

5

B is independently a nucleotide base or its analog or hydrogen;

X is independently a phosphorus-containing group; and

10

R<sub>2</sub> is independently blocking group or a phosphorus-containing group.

15

98. The compound of claim 97, wherein said compound is a nucleotide.
99. The compound of claim 97, wherein said compound is a nucleotide-tri-phosphate.
100. A polynucleotide comprising the compound of claim 97 at one or more positions.
101. The polynucleotide of claim 100, wherein said polynucleotide is an enzymatic nucleic acid.
102. The enzymatic nucleic acid of claim 101, wherein said nucleic acid is in a hammerhead configuration.
103. The enzymatic nucleic acid of claim 102, wherein said nucleic acid is in a hairpin configuration.
- 20 104. The enzymatic nucleic acid of claim 102, wherein said nucleic acid is in a hepatitis delta virus, group I intron, VS RNA, group II intron or RNase P RNA configuration.
105. The compound of claim 97, wherein said compound is xylo riboadenosine.

106. The compound of claim 97, wherein said compound is xylo riboguanosine.
107. The compound of claim 97, wherein said compound is xylo ribonucleoside phosphoramidite.
108. The compound of claim 107, wherein said compound is xylo riboguanosine phosphoramidite.
109. The compound of claim 107, wherein said compound is xylo riboadenosine phosphoramidite.
110. A mammalian cell comprising the compound of claim 97.
111. The mammalian cell of claim 14, wherein said cell is a human cell.
112. A mammalian cell comprising the compound of claim 101.
113. The mammalian cell of claim 112, wherein said cell is a human cell.
114. A method of making a polynucleotide of claim 100.
115. A method of modulating gene expression using a polynucleotide of claim 100.
116. A pharmaceutical composition comprising a compound of claim 97.
117. A pharmaceutical composition comprising a polynucleotide of claim 101.
118. The compound of claim 97, wherein said compound is used as an antiviral agent.
119. A process for the synthesis of a xylo ribonucleoside phosphoramidite comprising the steps of:
  - a) oxidation of a 2' and 5'-protected ribonucleoside using an oxidant followed by reduction using a reducing agent under conditions suitable for the formation of 2' and 5'-protected xylofuranosyl nucleoside; and

b) phosphorylation under conditions suitable for the formation of xylofuranosyl nucleoside phosphoramidite.;

120. The process of claim 119, wherein said oxidantion is carried out in the presence of chromium oxide, pyridine, and acetic anhydride.

5 121. The process of claim 119, wherein said oxidantion is carried out in the presence of dimethylsulfoxide and acetic anhydride.

122. The process of claim 119, wherein said oxidantion is carried out in the presence of Dess-Martin reagent (periodinane).

10 123. The process of claim 119, wherein said reduction is carried out in the presence of triacetoxy sodium borohydride.

124. The process of claim 119, wherein said reduction is carried out in the presence of sodium borohydride

125. The process of claim 119, wherein said reduction is carried out in the presence of lithium borohydride,

15 126. A process for one pot deprotection of RNA comprising protecting groups, comprising the steps of:

a) contacting said RNA with a mixture of anhydrous alkylamine, trialkylamine and a polar organic reagent in a predetermined proportions, at room temperature for about between 30 and 100 min under conditions suitable for the removal of nucleic acid base and phosphate protecting groups from said RNA; and

20 b) contacting the resulting RNA from step a with an anhydrous triethylamine-hydrogen fluoride at about between 50 °C-70 °C under conditions suitable for the removal of a 2'-OH protecting group.

127. The process of claim 126 wherein said RNA is an enzymatic RNA molecule.

128. The process of claim 128, wherein said enzymatic RNA molecule is in a hammerhead motif.

129 The process of claim 126, wherein said polar organic reagent is dimethylsulfoxide

130. The process of claim 126, wherein said anhydrous alkylamine is anhydrous methyl  
5 amine.

131. The process of claim 126, wherein said anhydrous alkylamine is anhydrous ethylamine.

132. The process of claim 126, wherein said trialkylamine is triethylamine.

133. The process of claim 129, wherein the predetermined proportion of anhydrous  
10 alkylamine, trialkylamine and dimethylsulfoxide in said mixture is 10, 3 and 13,  
respectively.

134. A process for one pot deprotection of RNA comprising protecting groups,  
comprising the steps of:

15 a) contacting said RNA with a mixture of anhydrous methylamine, triethylamine  
and dimethylsulfoxide in proportions of 10, 3 and 13, respectively, at room  
temperature for about 90 min under conditions suitable for the removal of nucleic  
acid base and phosphate protecting groups from said RNA; and

20 b) contacting the resulting RNA from step a with an anhydrous  
triethylamine-hydrogen fluoride at about between 65°C under conditions suitable  
for the removal of a 2'-OH protecting group.

135 The process of claim 134 wherein said RNA is an enzymatic RNA molecule.

136. The process of claim 135, wherein said enzymatic RNA molecule is in a  
hammerhead motif.

137 A process for one pot deprotection of RNA comprising protecting groups, comprising the steps of:

5 a) contacting said RNA with a mixture of anhydrous alkylamine and a polar organic reagent in a predetermined proportions, at room temperature for about between 30 and 100 min under conditions suitable for the removal of nucleic acid base and phosphate protecting groups from said RNA; and

b) contacting the resulting RNA with a anhydrous triethylamine•hydrogen fluoride at about between 50 °C-70 °C under conditions suitable for the removal of 2'-OH protecting group.

10 138. The process of claim 137 wherein said RNA is an enzymatic RNA molecule.

139. The process of claim 138, wherein said enzymatic RNA molecule is in a hammerhead motif.

140. The process of claim 137, wherein said polar organic reagent is dimethylsulfoxide

141. The process of claim 137, wherein said anhydrous alkylamine is anhydrous methyl  
15 amine.

142. The process of claim 137, wherein said anhydrous alkylamine is anhydrous ethylamine.

143. An nucleic acid catalyst with RNA cleaving activity, wherein said nucleic acid catalyst modulates the expression of a Raf gene.

20 144. The nucleic acid catalyst of claim 143, wherein said nucleic acid catalyst is in a hammerhead configuration.

145. The nucleic acid catalyst of claim 144, wherein said nucleic acid catalyst comprises a stem II region of length greater than or equal to 2 base pairs.

146. The nucleic acid catalyst of claim 143, wherein said nucleic acid catalyst is in a hairpin configuration.
147. The nucleic acid catalyst of claim 143, wherein said enzymatic nucleic acid is in a hepatitis  $\delta$  virus, group I intron, group II intron, VS nucleic acid or RNase P nucleic acid configuration.  
5
148. The enzymatic nucleic acid of claim 146, wherein said nucleic acid catalyst comprises a stem II region of length between three and seven base-pairs.
149. The nucleic acid catalyst of claim 143, wherein said nucleic acid comprises between 12 and 100 bases complementary to said RNA.
- 10 150. The nucleic acid catalyst of claim 143, wherein said nucleic acid comprises between 14 and 24 bases complementary to said mRNA.
151. The nucleic acid catalyst of claim 144, wherein said nucleic acid catalyst consists essentially of any sequence defined as Seq ID Nos 502-1102, 1153-1460 and 1913-2353.
- 15 152. A mammalian cell including an nucleic acid catalyst of any of claim 143.
153. The mammalian cell of claim 152, wherein said mammalian cell is a human cell.
154. An expression vector comprising nucleic acid sequence encoding at least one nucleic acid catalyst of claim 143, in a manner which allows expression of that nucleic acid catalyst.
- 20 155. A mammalian cell including an expression vector of claim 154.
156. The mammalian cell of claim 155, wherein said mammalian cell is a human cell.
157. A method for treatment of cancer, restenosis, psoriasis and rheumatoid arthritis comprising the step of administering to a patient the nucleic acid catalyst of claim 143.

158. A method for treatment of cancer, restenosis, psoriasis and rheumatoid arthritis comprising the step of administering to a patient the expression vector of claim 154.
159. A method for treatment of cancer comprising the steps of: a) isolating cells from a patient; b) administering to said cells the nucleic acid catalyst of claim 143; and c) introducing said cells back into said patient.  
5
160. A pharmaceutical composition comprising the nucleic acid catalyst of claim 143.
161. A method of treatment of a patient having a condition associated with the level of c-raf, wherein said patient is administered the nucleic acid catalyst of claim 143.  
10
162. A method of treatment of a patient having a condition associated with the level of c-raf, comprising contacting cells of said patient with the nucleic acid molecule of claim 143, and further comprising the use of one or more drug therapies.
163. The nucleic acid catalyst of claim 144, wherein said nucleic acid molecule comprises at least five ribose residues, and wherein said nucleic acid comprises phosphorothioate linkages at at least three of the 5' terminal nucleotides, and wherein said nucleic acid comprises a 2'-C-allyl modification at position No. 4 of said nucleic acid, and wherein said nucleic acid comprises at least ten 2'-O-methyl modifications, and wherein said nucleic acid comprises a 3'- end modification.  
15
164. The enzymatic nucleic acid of claim 163, wherein said nucleic acid comprises a 3'-3' linked inverted ribose moiety at said 3' end.  
20
165. The nucleic acid catalyst of claim 144, wherein said nucleic acid molecule comprises at least five ribose residues, and wherein said nucleic acid molecule comprises phosphorothioate linkages at at least three of the 5' terminal nucleotides, and wherein said nucleic acid comprises a 2'-amino modification at position No. 4 and/or at position No. 7 of said nucleic acid molecule, wherein said  
25

nucleic acid molecule comprises at least ten 2'-O-methyl modifications, and wherein said nucleic acid comprises a 3'- end modification.

166. The nucleic acid catalyst of claim 144, wherein said nucleic acid molecule comprises at least five ribose residues, and wherein said nucleic acid molecule comprises phosphorothioate linkages at at least three of the 5' terminal nucleotides, and wherein said nucleic acid molecule comprises an abasic substitution at position No. 4 and/or at position No. 7 of said nucleic acid molecule, wherein said nucleic acid comprises at least ten 2'-O-methyl modifications, and wherein said nucleic acid molecule comprises a 3'-end modification.

167. The nucleic acid catalyst of claim 144, wherein said nucleic acid molecule comprises of at least five ribose residues, and wherein said nucleic acid comprises phosphorothioate linkages at at least three of the 5' terminal nucleotides, and wherein said nucleic acid molecule comprises a 6-methyl uridine substitution at position No. 4 and/or at position No. 7 of said nucleic acid molecule, wherein said nucleic acid molecule comprises at least ten 2'-O-methyl modifications, and wherein said nucleic acid molecule comprises a 3' end modification.

168. A method for modulating expression of c-raf gene in a mammalian cell by administering to said cell the nucleic acid catalyst of claim 143.

169. A method of cleaving a separate RNA molecule comprising, contacting the nucleic acid catalyst of claim 143 with said separate RNA molecule under conditions suitable for the cleavage of said separate RNA molecule.

170. The method of claim 169, wherein said cleavage is carried out in the presence of a divalent cation.

171. The method of claim 170, wherein said divalent cation is  $Mg^{2+}$ .

172. The nucleic acid molecule of claim 143, wherein said nucleic acid is chemically synthesized.

173. The expression vector of claim 154, wherein said vector comprises:

- 5 a) a transcription initiation region;
- b) a transcription termination region;
- c) a gene encoding at least one said nucleic acid molecule; and

wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

10 174. The expression vector of claim 154, wherein said vector comprises:

- a) a transcription initiation region;
- b) a transcription termination region;
- c) an open reading frame;

15 d) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and

wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

175. The expression vector of claim 154, wherein said vector comprises:

20

- a) a transcription initiation region;
- b) a transcription termination region;
- c) an intron;

d) a gene encoding at least one said nucleic acid molecule; and

wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

5 176. The expression vector of claim 154, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) an intron;

d) an open reading frame;

10 e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and

wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

15 177. The nucleic acid catalyst of claim 144, wherein said enzymatic nucleic acid comprises sequences that are complementary to any of sequences defined as Seq ID Nos 1-501, 1461-1768 and 2354-2794.

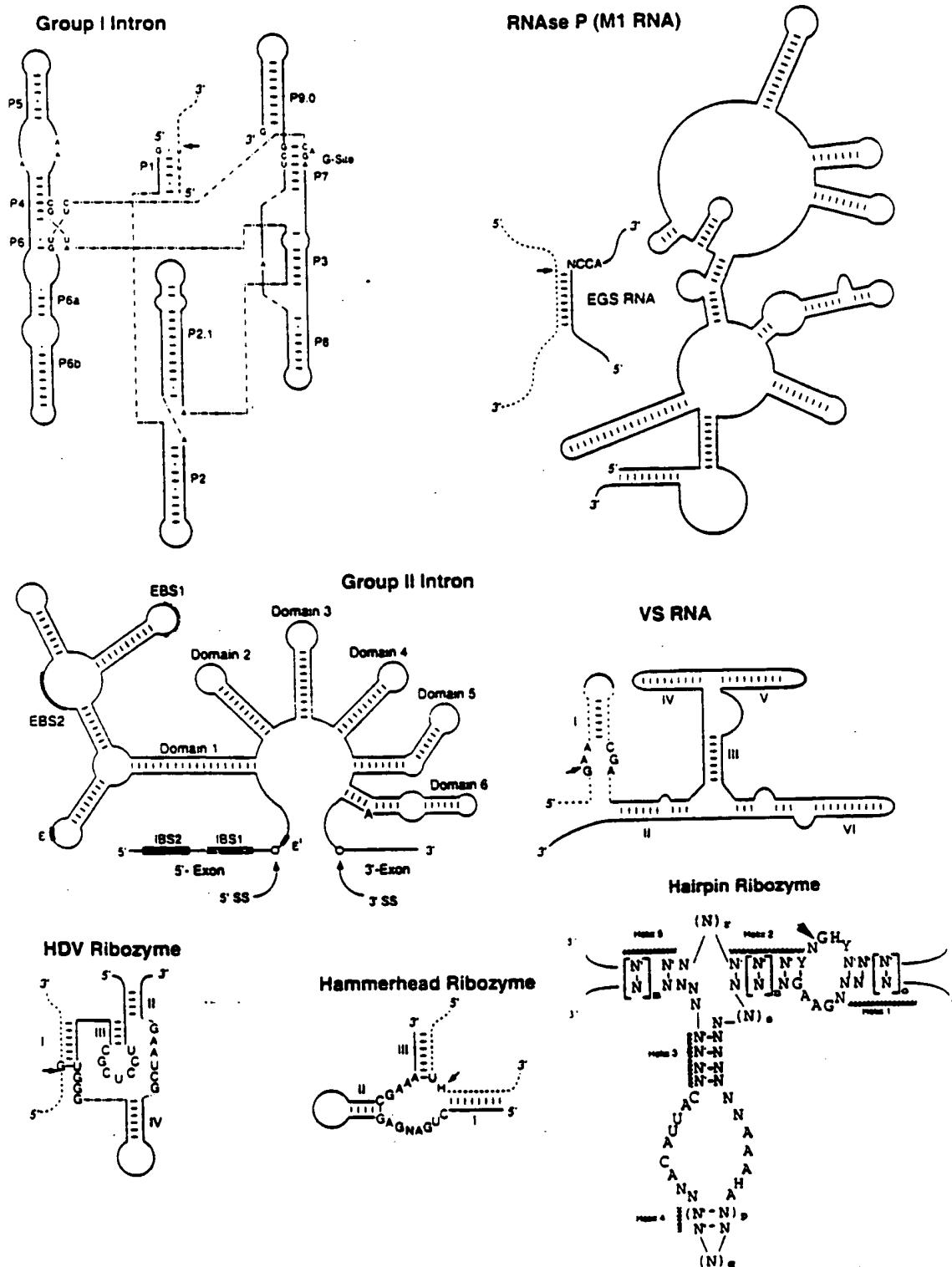
178. The nucleic acid catalyst of claim 146, wherein said nucleic acid catalyst consists essentially of any sequence defined as Seq ID Nos 1003-1077, 1769-1840 and  
20 2795-2845.

179. The nucleic acid catalyst of claim 146, wherein said enzymatic nucleic acid comprises sequences that are complementary to any of sequences defined as Seq ID Nos 1078-1152, 1841-1912 and 2846-2896.

180. The nucleic acid catalyst of claim 144, wherein said enzymatic nucleic acid comprises sequences complementary to any of sequences defined as Seq ID Nos 2897-2956.
181. The nucleic acid catalyst of claim 143, wherein said Raf gene is c-Raf-1 gene.
- 5 182. The nucleic acid catalyst of claim 143, wherein said Raf gene is A-Raf gene.
183. The nucleic acid catalyst of claim 143, wherein said Raf gene is B-Raf gene.
184. The nucleic acid catalyst of claim 143, wherein said enzymatic nucleic acid is a DNA enzyme.
185. The nucleic acid catalyst of claim 143, wherein said enzymatic nucleic acid 10 comprises at least one 2'-sugar modification.
186. The nucleic acid catalyst of claim 143, wherein said enzymatic nucleic acid comprises at least one nucleic acid base modification.
187. The nucleic acid catalyst of claim 143, wherein said enzymatic nucleic acid comprises at least one phosphorothioate modification.
- 15 188. A method of treatment of a systemic disease in a patient comprising the step of systemically administering to said patient a nucleic acid catalyst which specifically cleaves RNA associated with said disease, under conditions in which said RNA in said patient is cleaved and a therapeutic result is attained.
189. The method of claim 188, wherein said disease is selected from the group 20 consisting of cancer, inflammation, psoriasis, non-hepatic ascites and infectious disease.
190. The method of claim 189, wherein said treatment of said cancer is characterised by a decrease in tumor metastasis.

191. The method of claim 189, wherein said treatment of said cancer is characterised by a decrease in tumor volume.
192. The method of claim 189, wherein said treatment of said cancer is characterised by a decrease in the progression of primary tumor.
- 5 193. The method of claim 188, wherein said nucleic acid catalyst is chemically modified.
194. The method of claim 188, wherein said nucleic acid catalyst is in a hammerhead motif.
- 10 195. The method of claim 194, wherein said hammerhead nucleic acid catalyst comprises 2'-C-allyl modification at position 4, phosphorothioate linkages at four 5'-terminal positions and inverted abasic nucleotide at the 3'-end of said nucleic acid molecule.
196. The method of claim 188, wherein said systemic administration is by intravenous administration of said nucleic acid catalyst into said patient.
- 15 197. The method of claim 188, wherein said systemic administration is by a bolus administration of said nucleic acid catalyst into said patient.
198. The method of claim 188, wherein said systemic administration is by continuous infusion of said nucleic acid catalyst into said patient.

**Figure 1: Ribozyme Motifs**



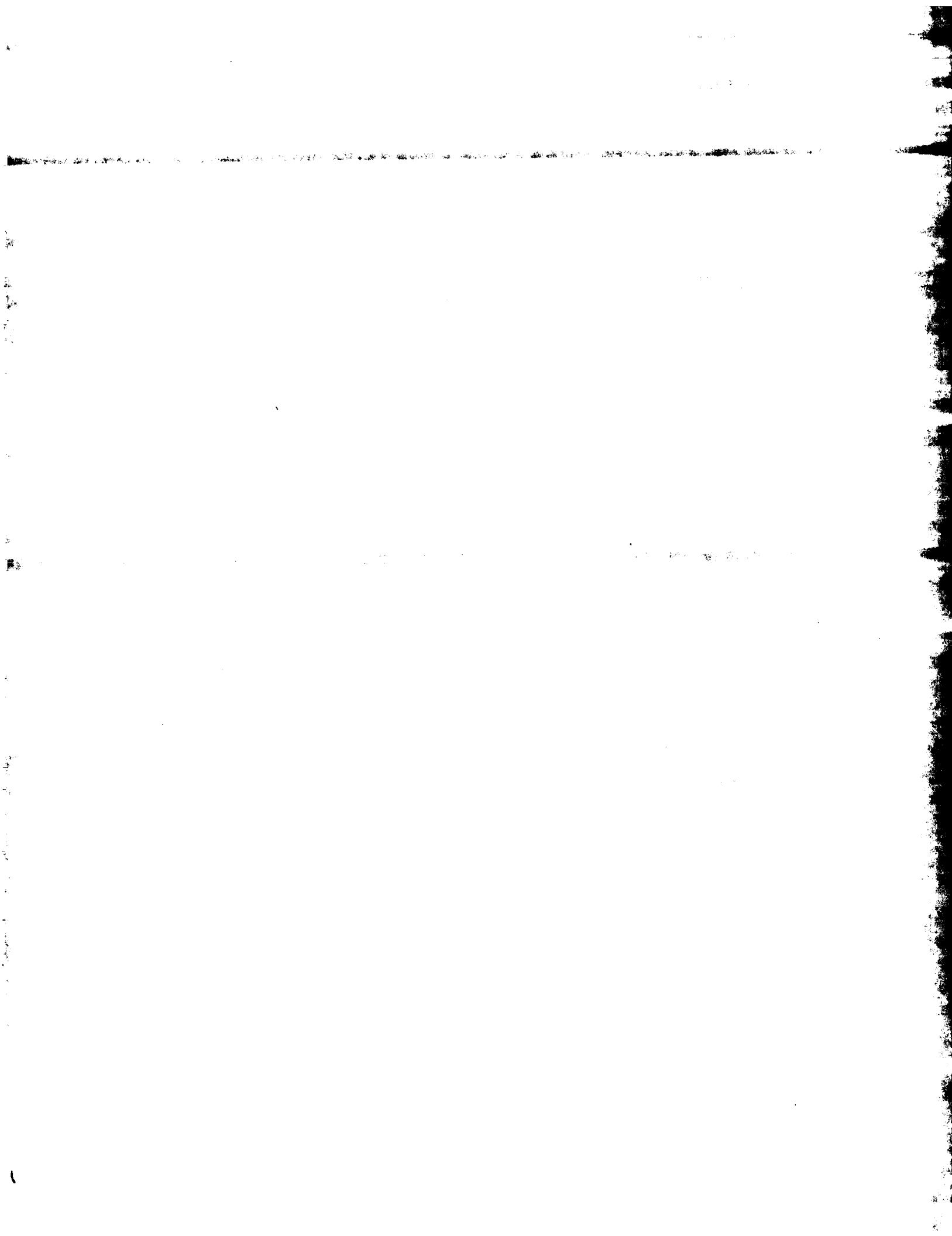
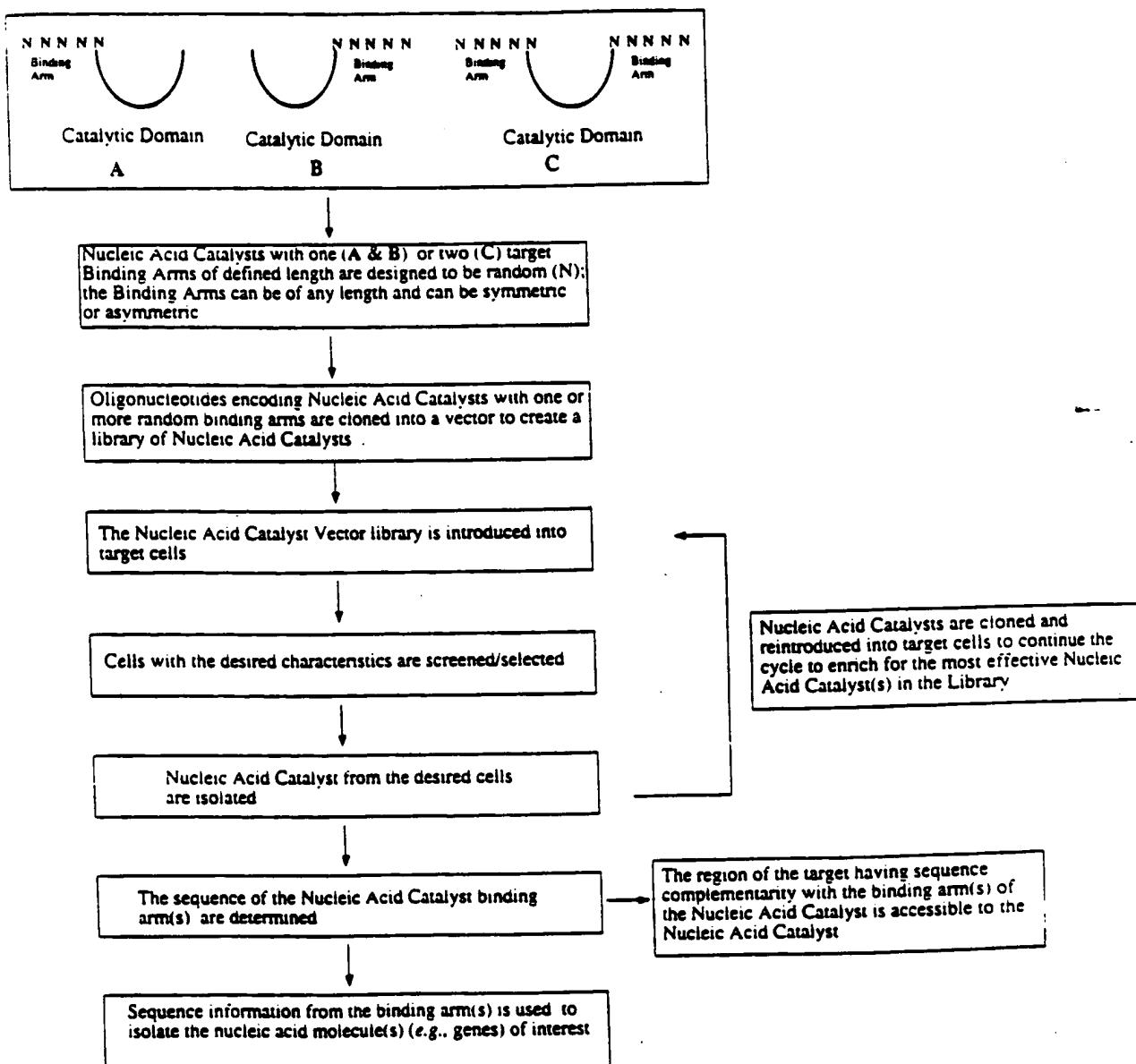
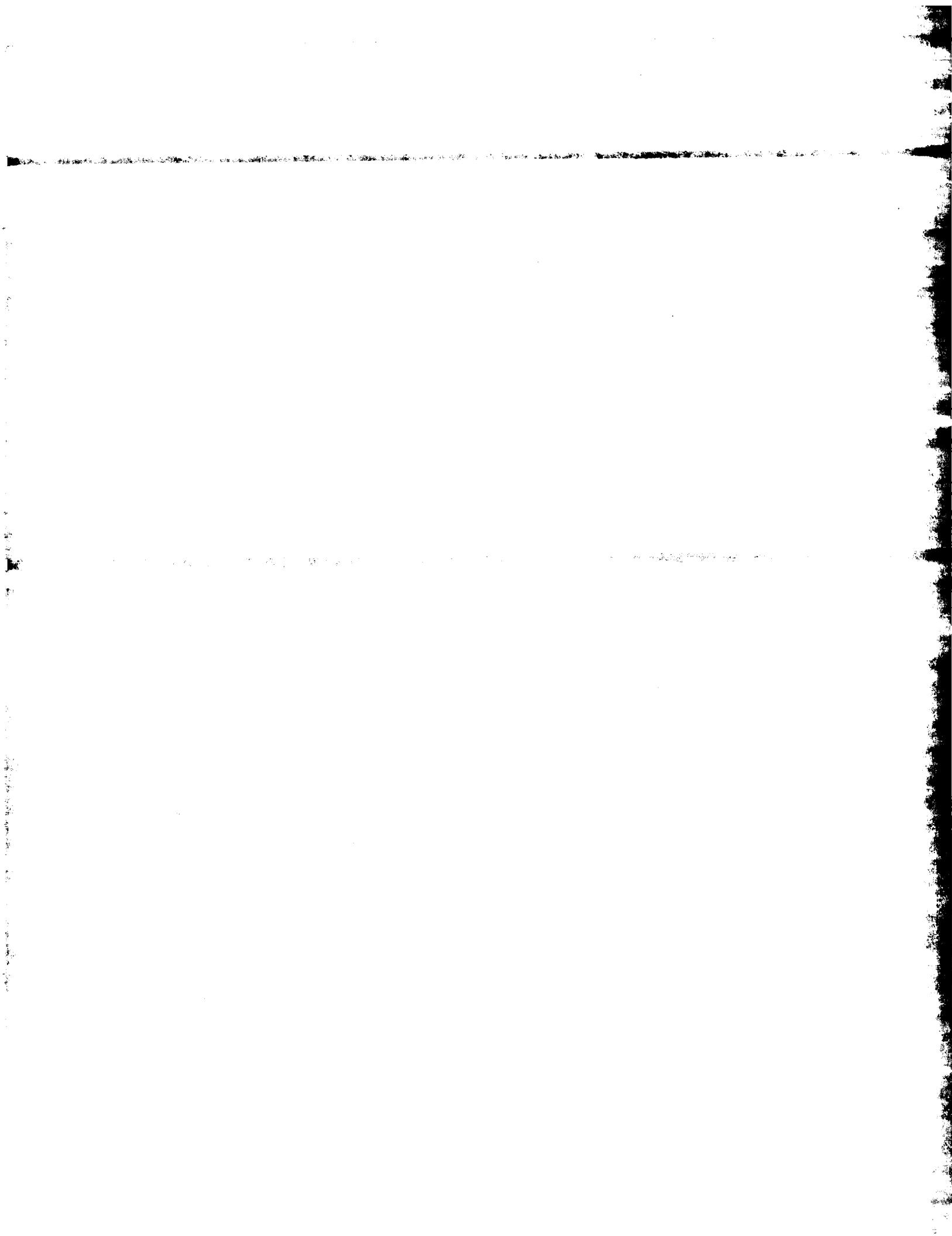
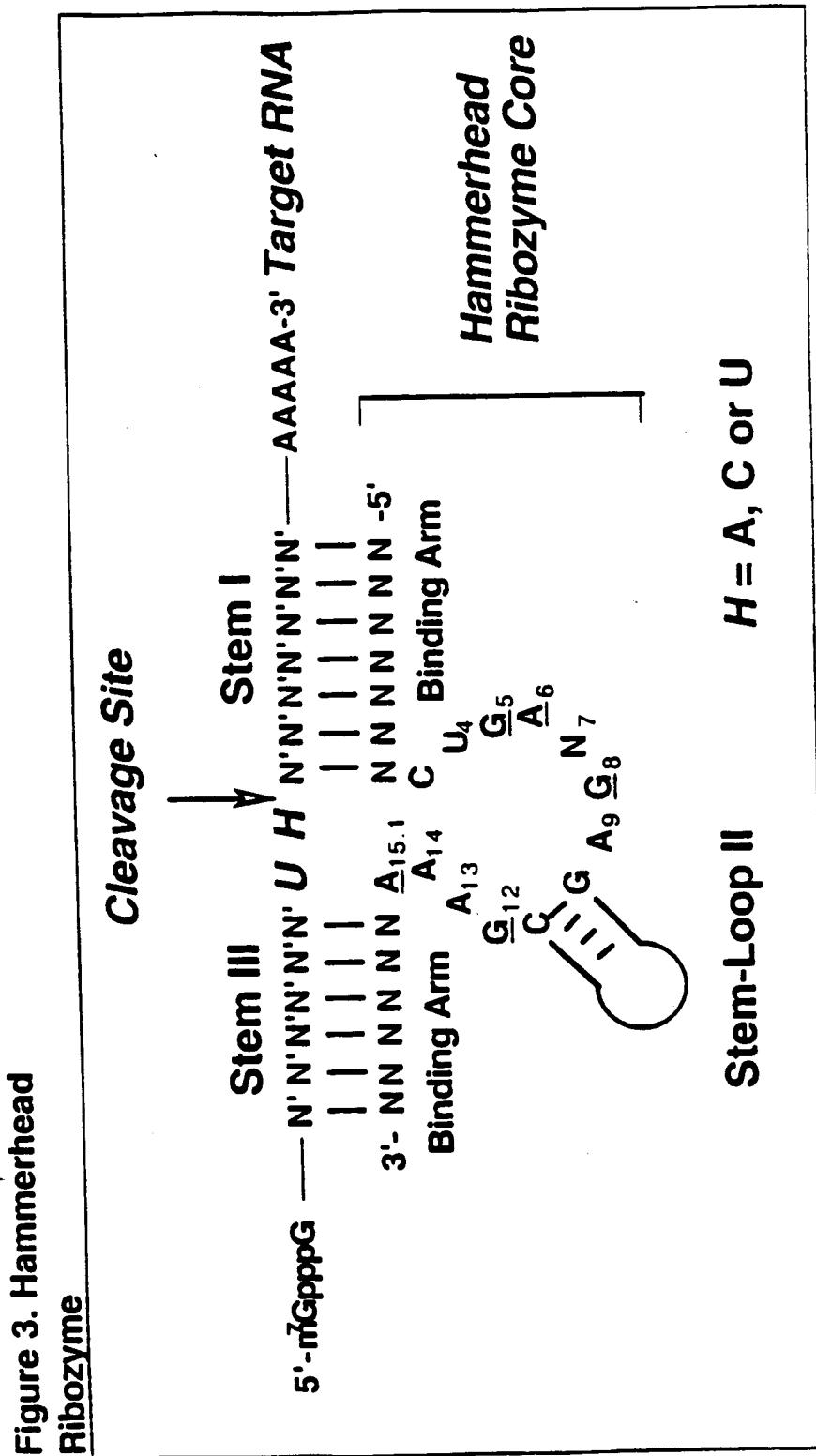
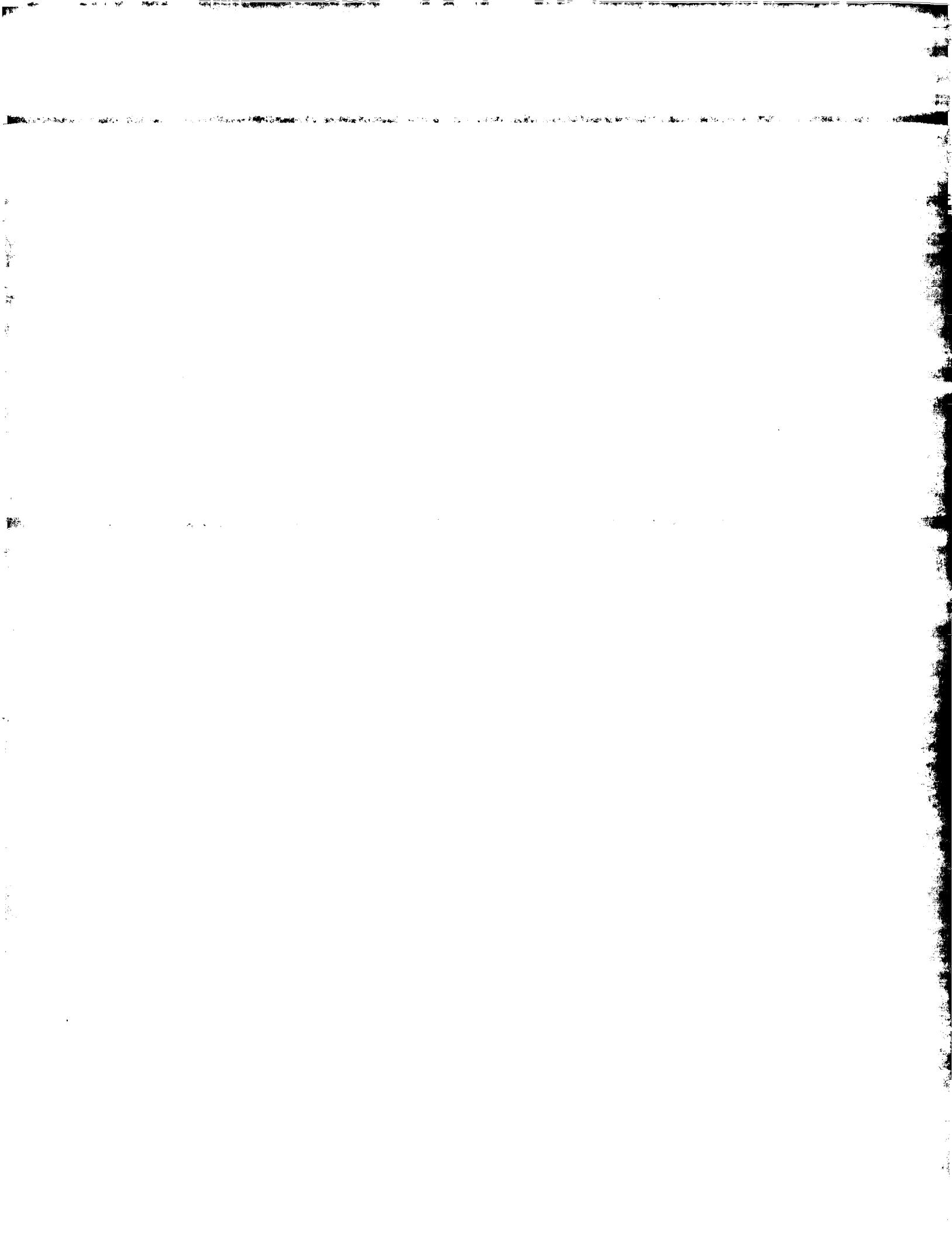


Figure 2: Accessible Site and Target Discovery

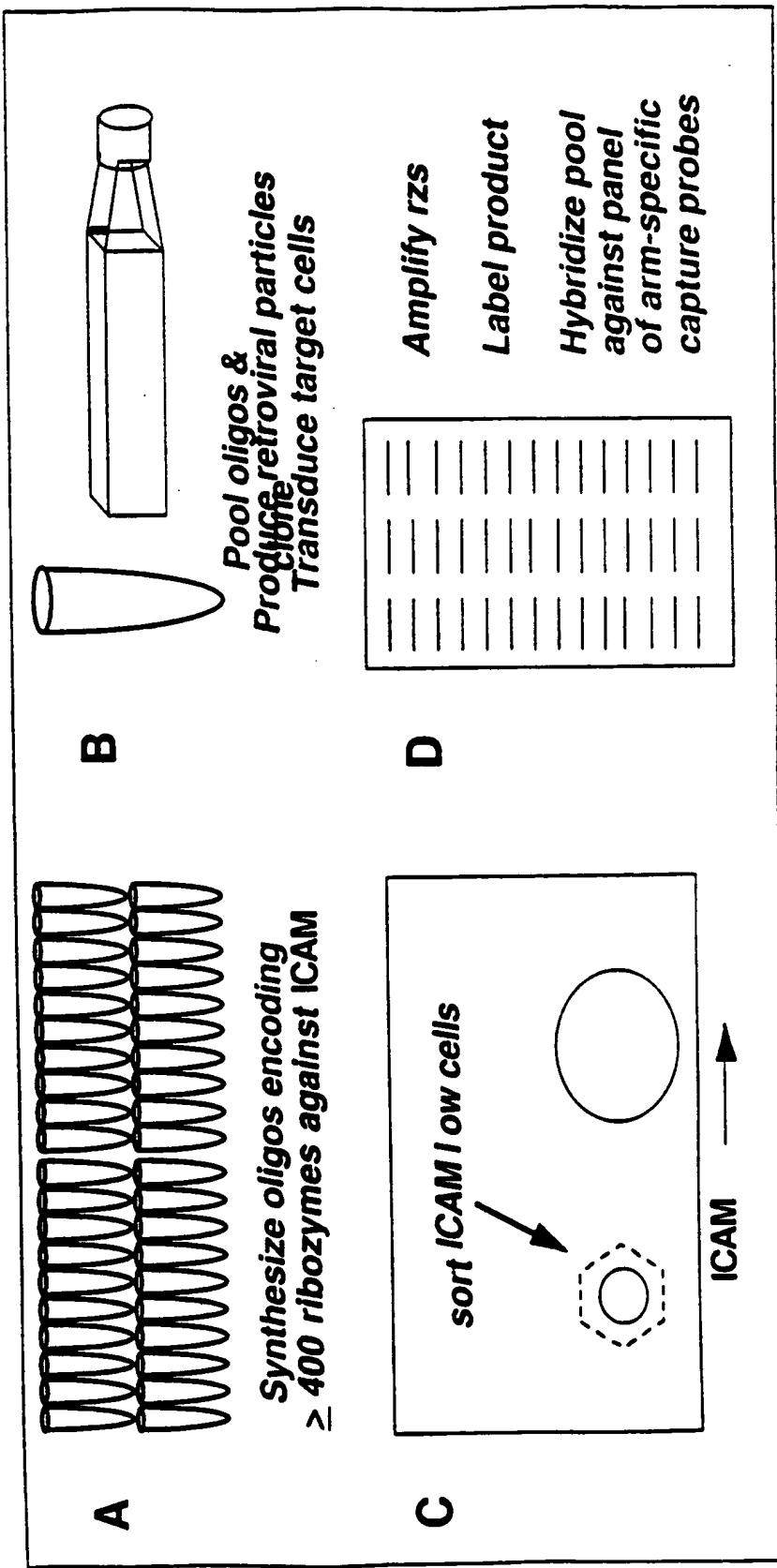


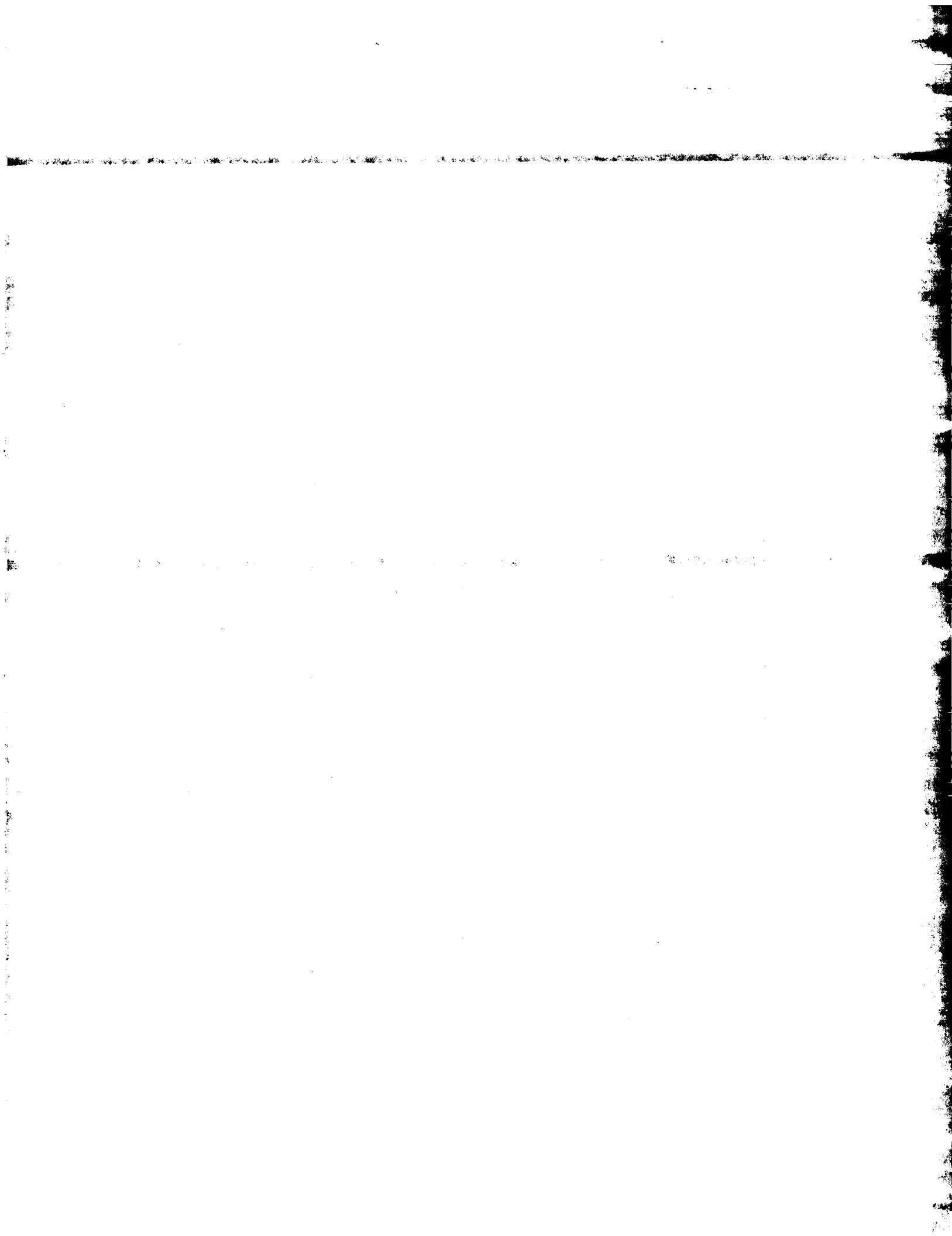




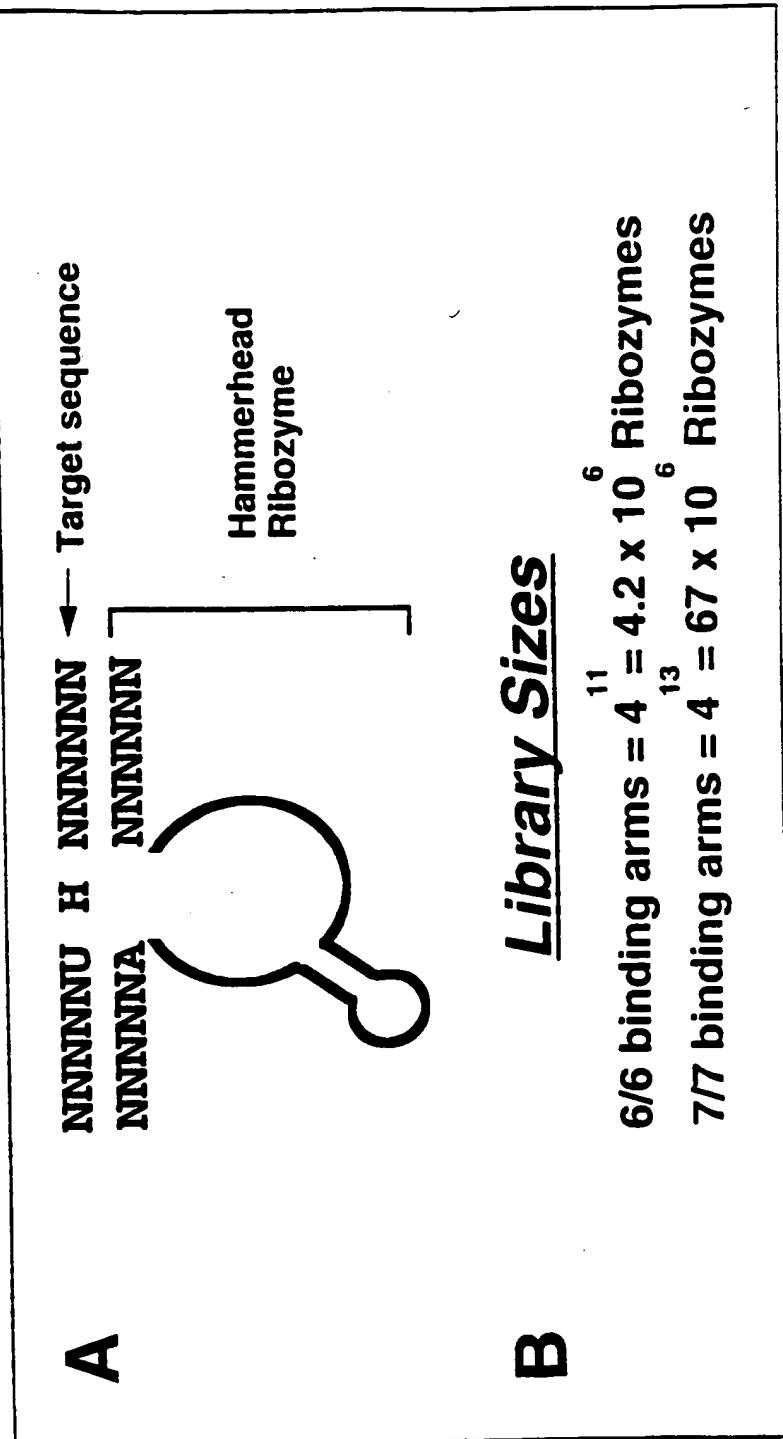


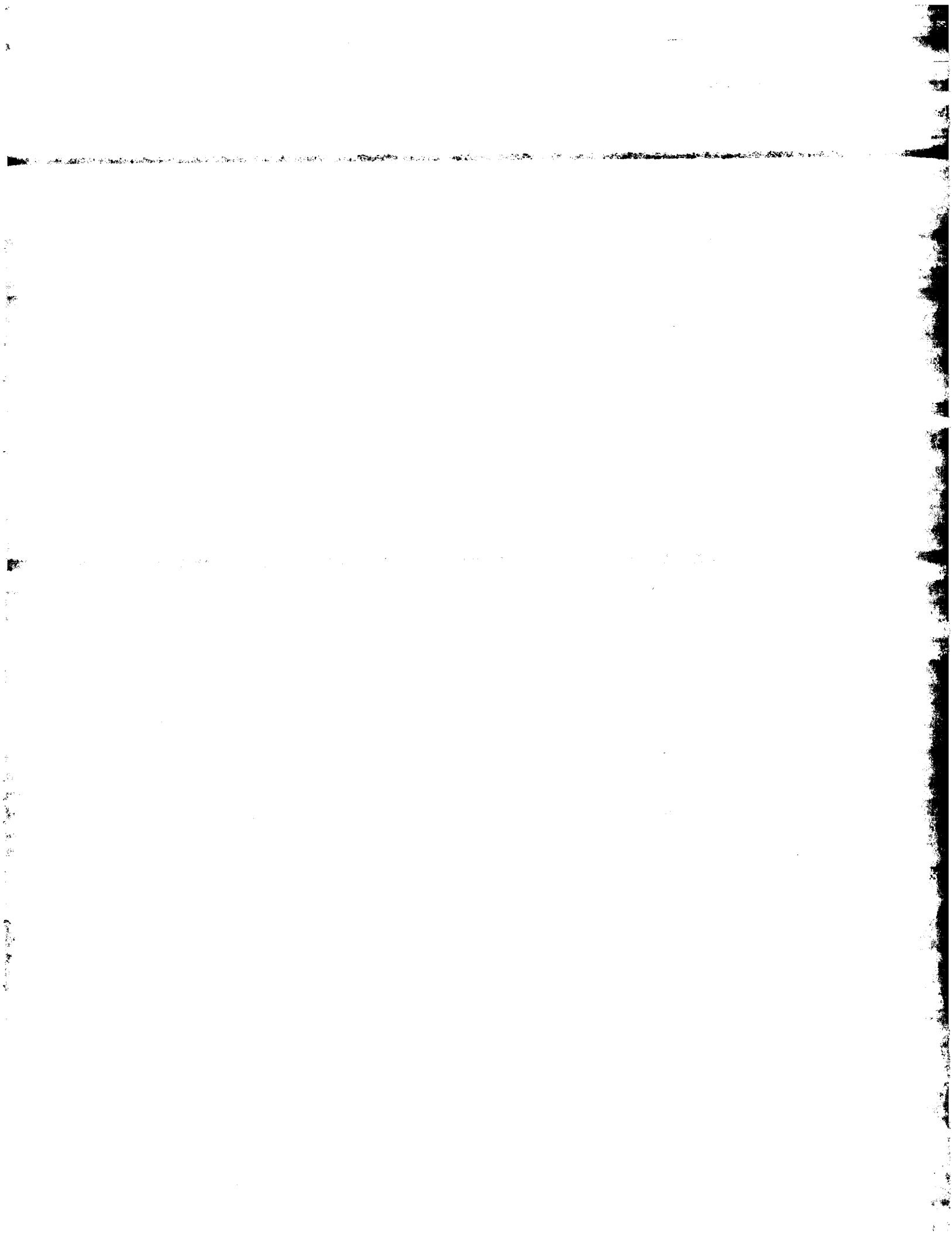
**Figure 4. A Scheme for the Synthesis of Defined Ribozyme Libraries**



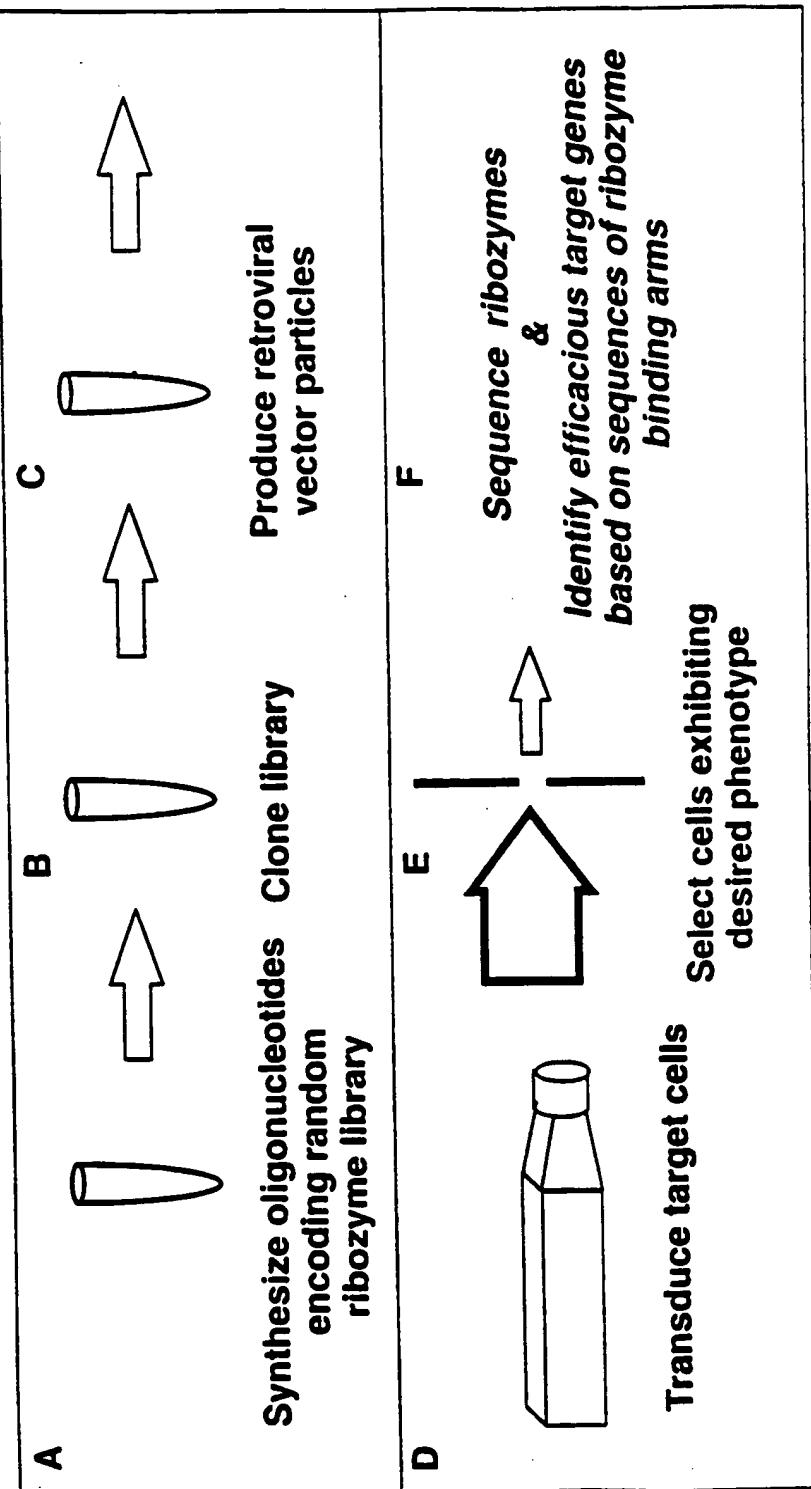


**Figure 5. Design and Synthesis of a Random Hammerhead Ribozyme Library (A), and Theoretical Complexities of a Random Hammerhead Library With Different Arm-Lengths (B)**



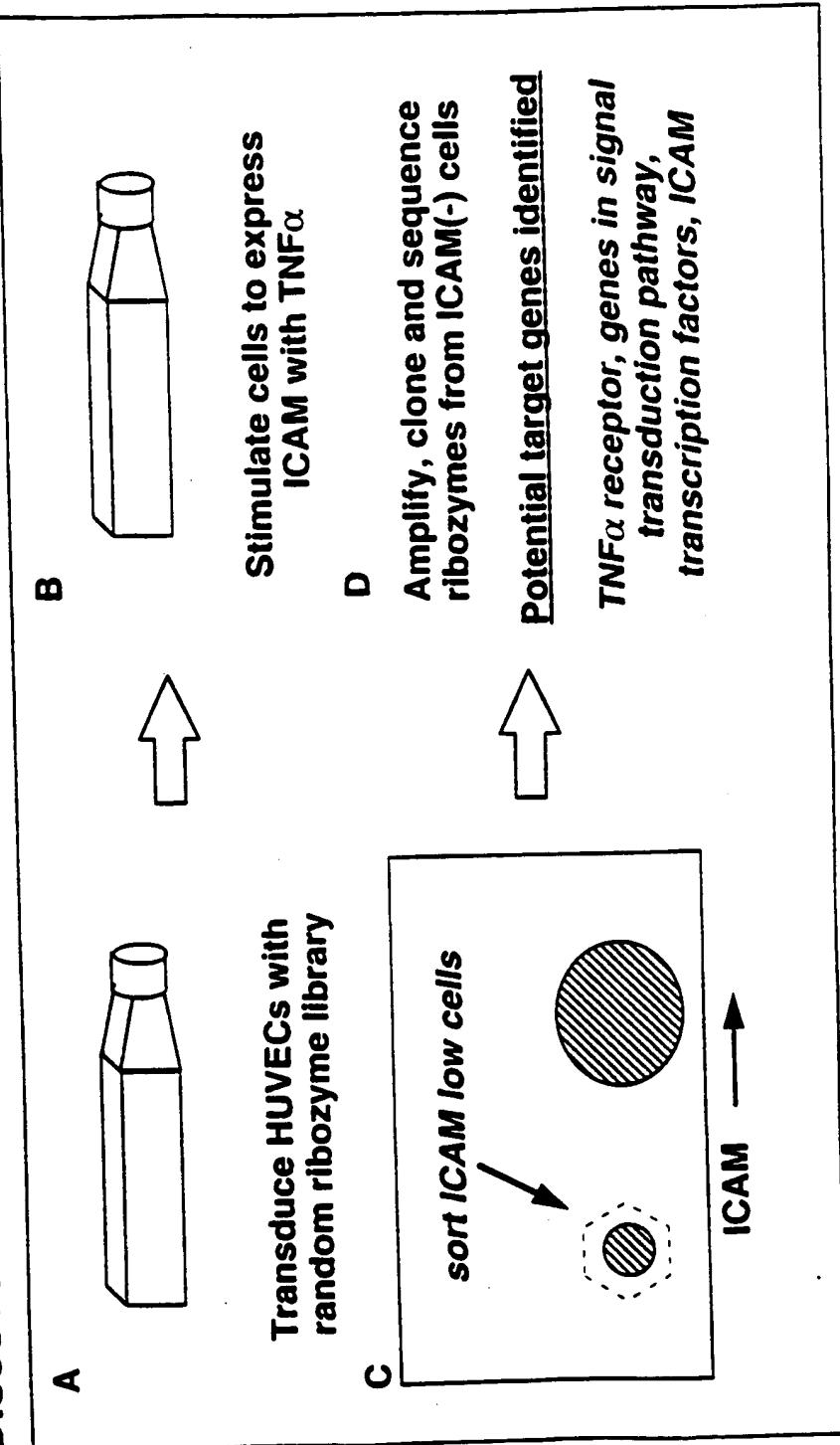


**Figure 6. Strategy for Discovery of Therapeutic Gene Targets Using Random Ribozyme Libraries**



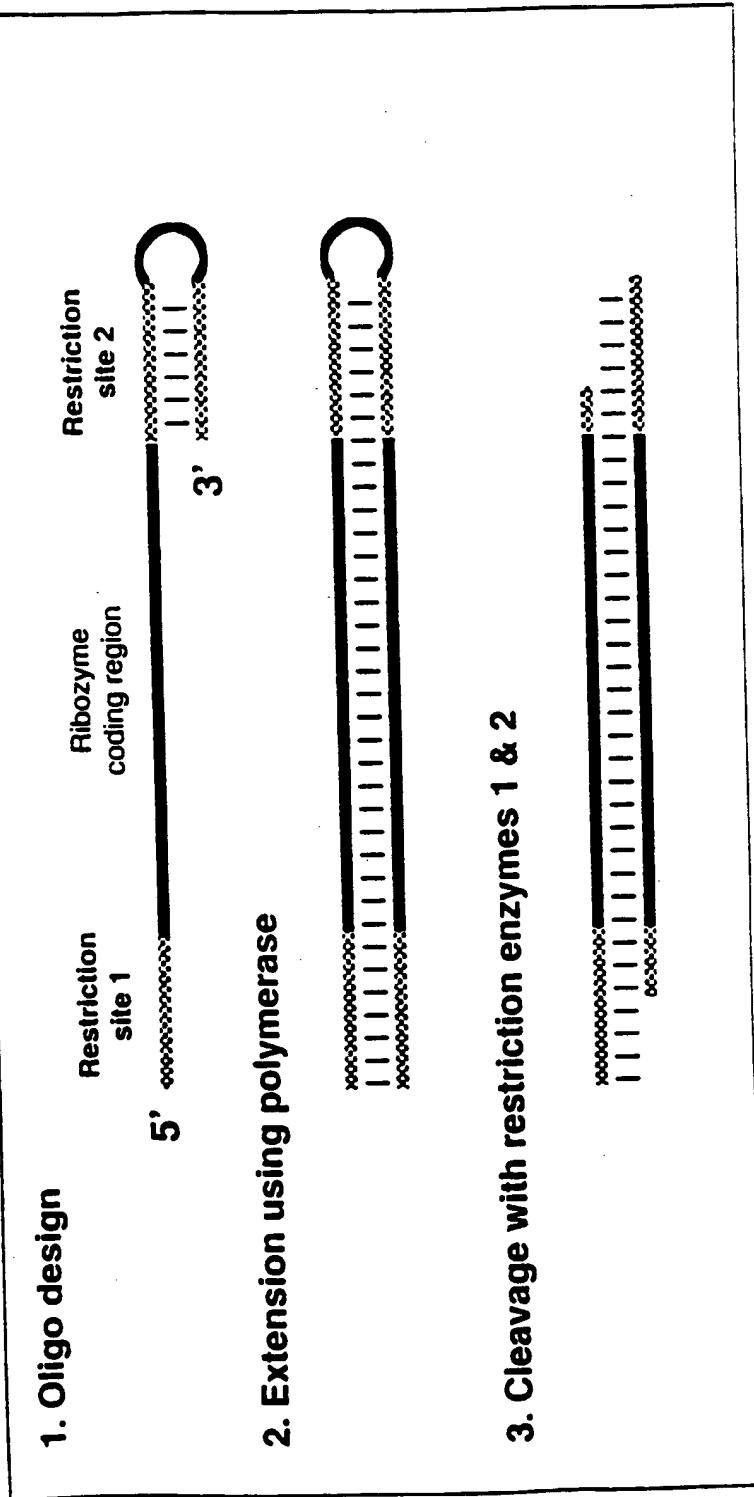


**Figure 7. Example of How Random Ribozyme Libraries Could Be used To Discover Critical Genes Involved in ICAM Expression**



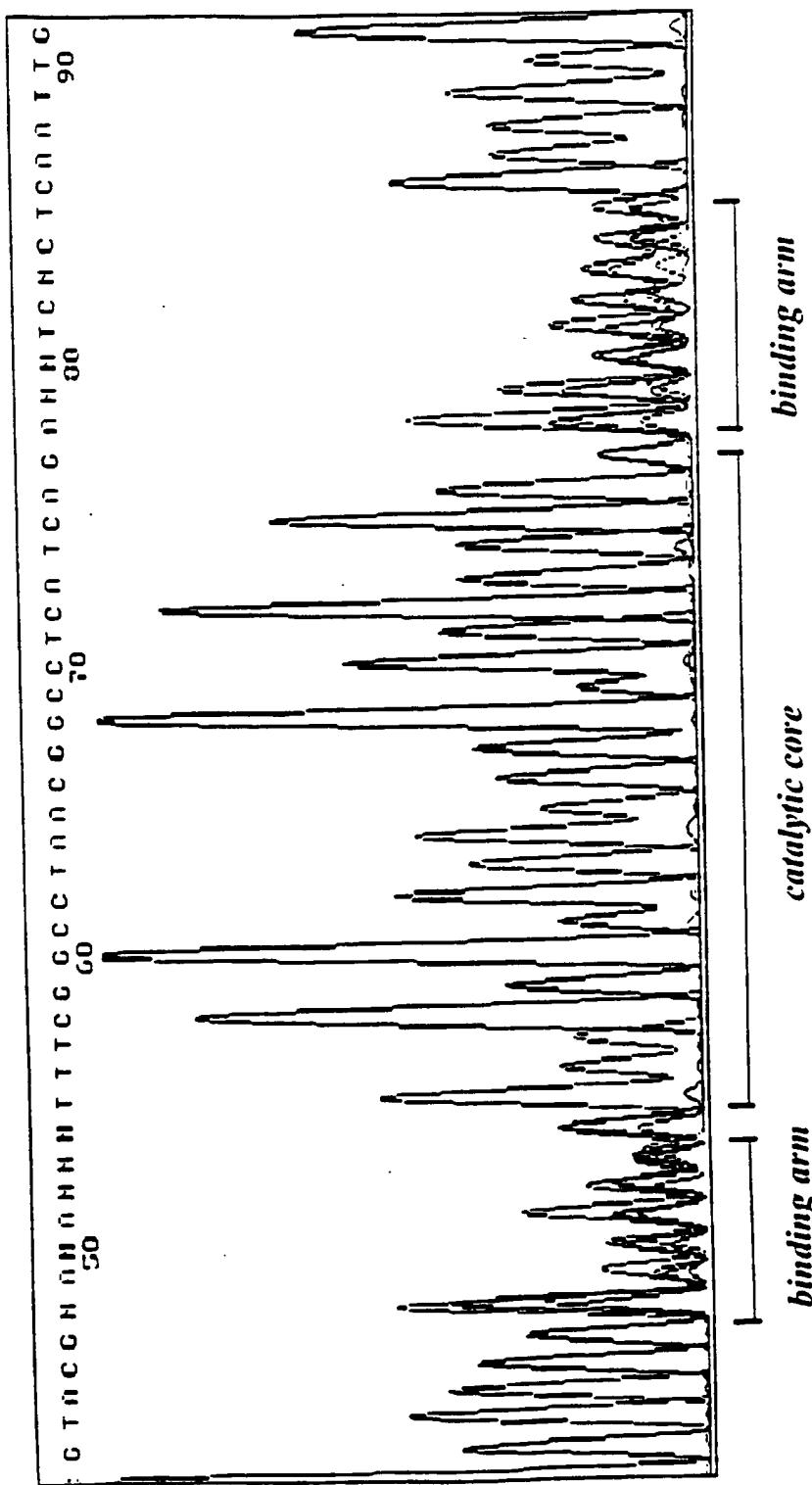


**Figure 8. Ribozyme Cloning Strategy: Design of Oligonucleotides Encoding Ribozyme Sequences and Preparation for Cloning**



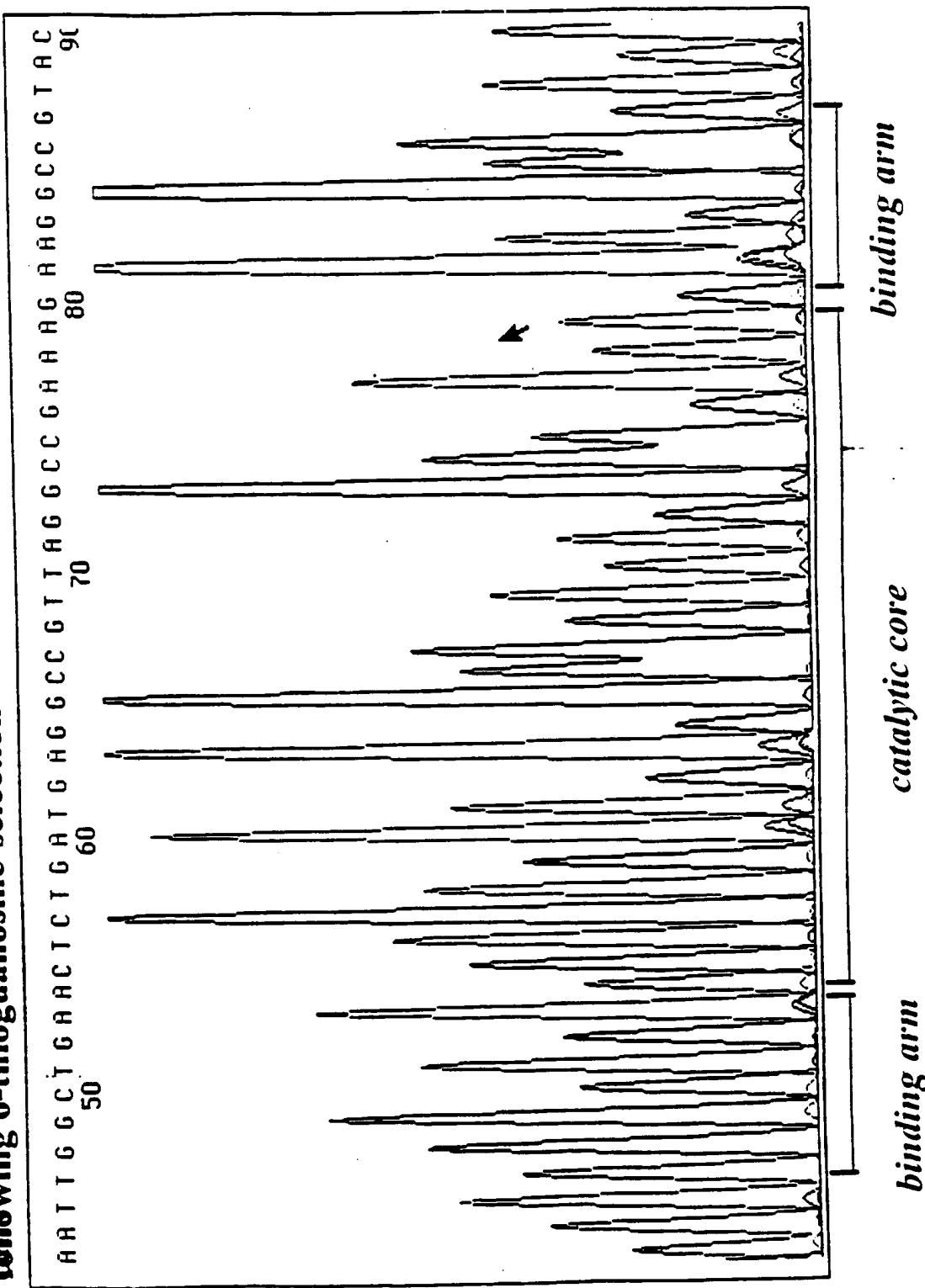


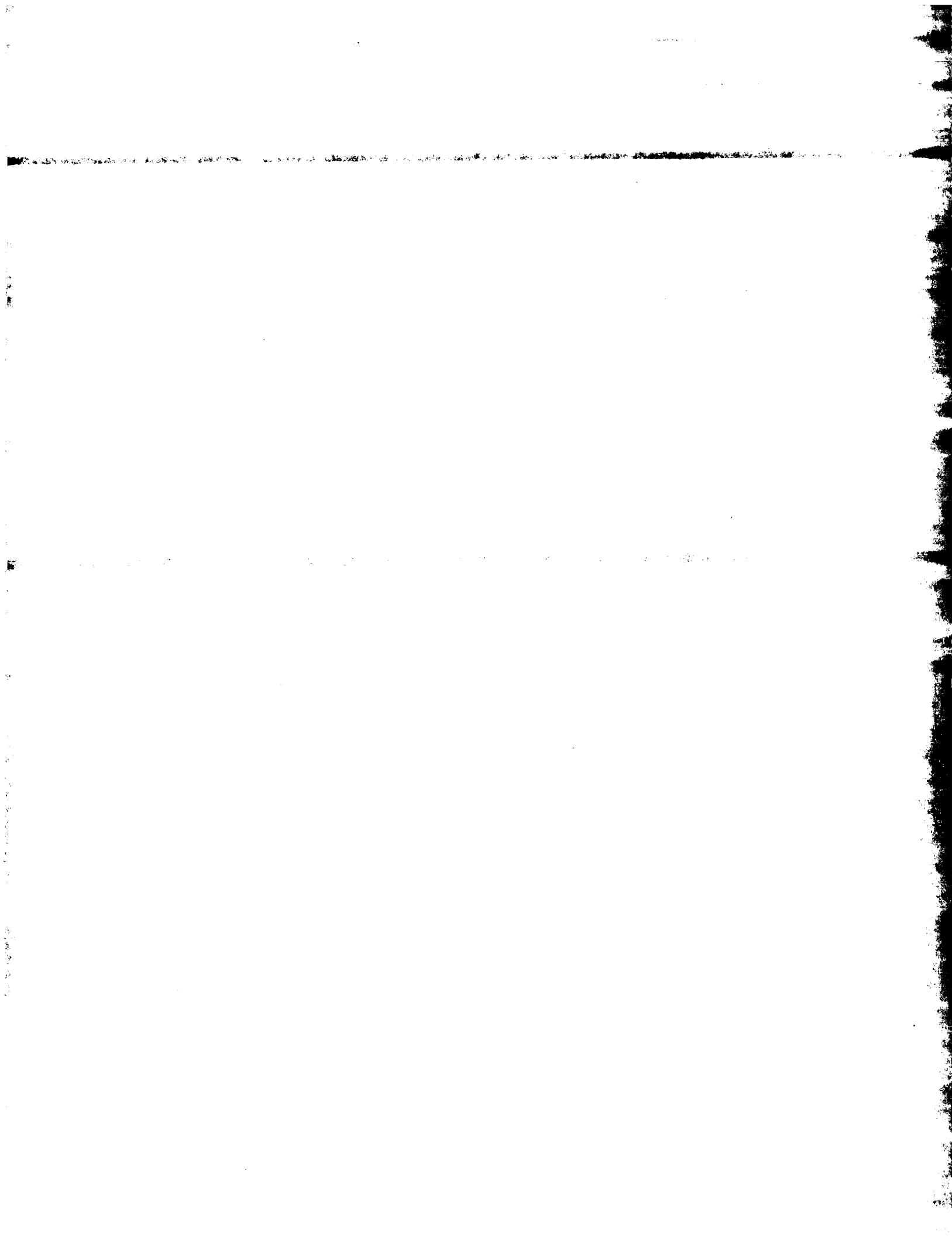
**Figure 9. Sequence Analysis of a Ribozyme Expression Vector Library**



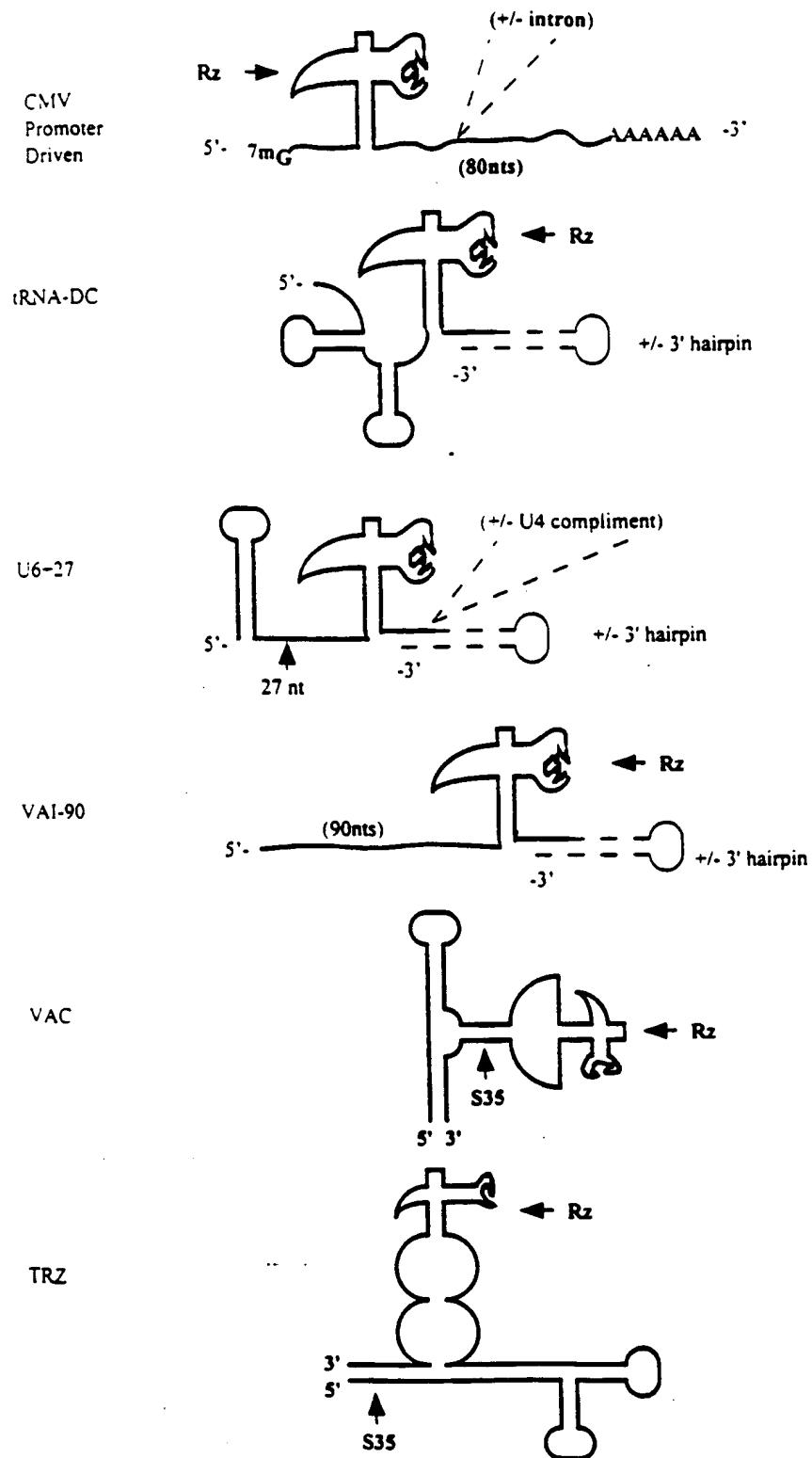


**Figure 10. Sequence analysis of PNP-targeted ribozymes in SupT1 human T following 6-thioguanosine-selection**





## Figure 11A: Pol II and Pol III RZ Constructs



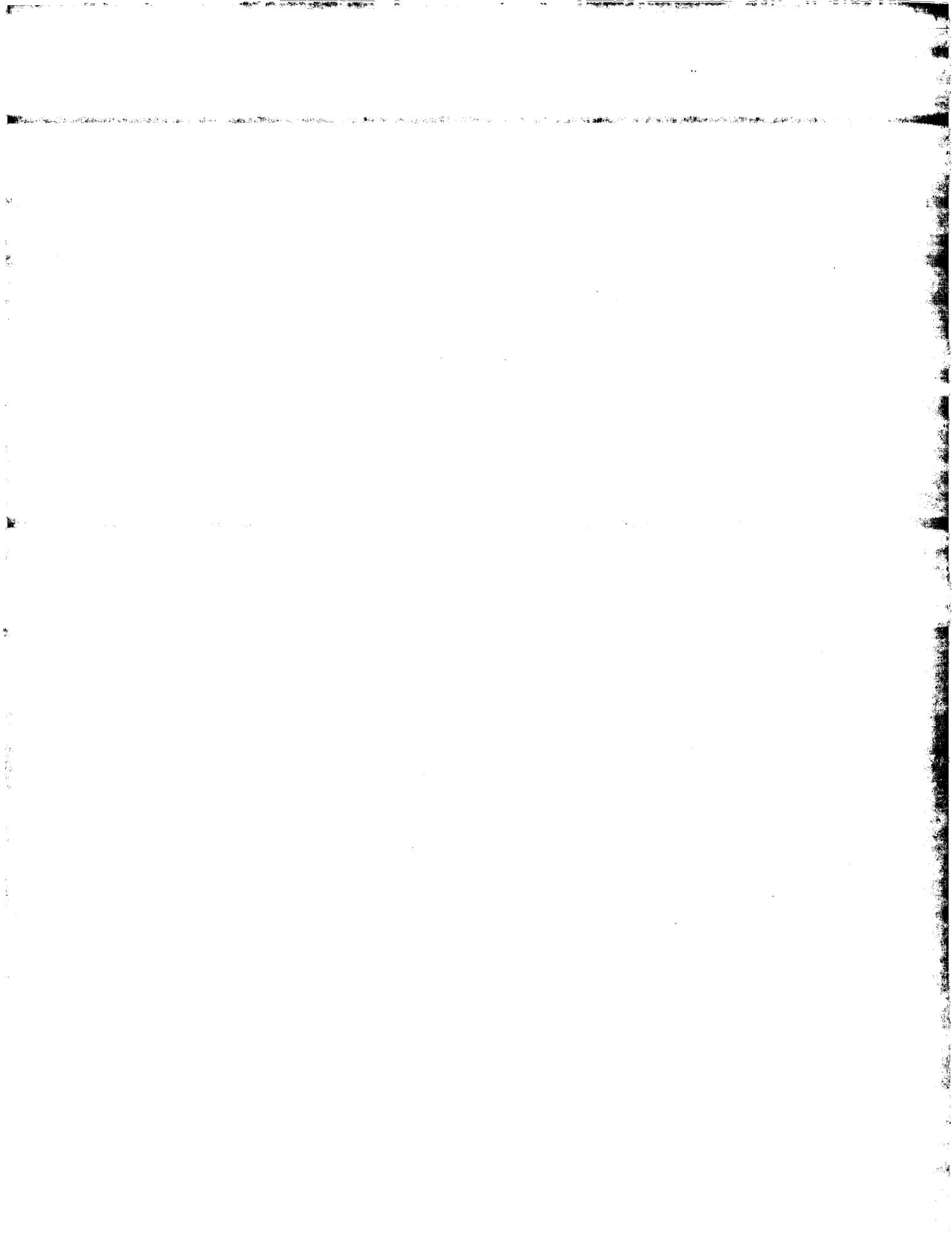
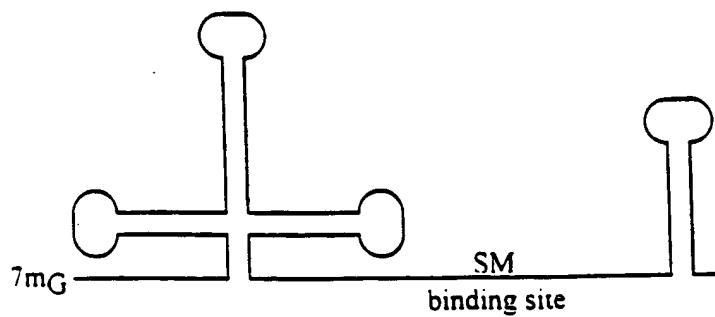
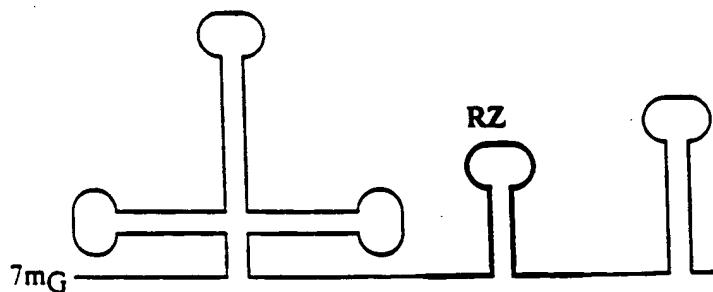


Figure 11B: Ribozyme transcription Units Based on U1 snRNA

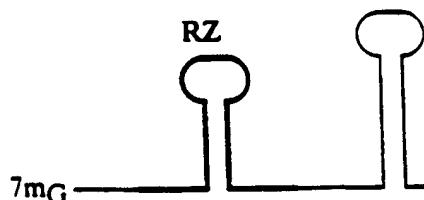


Wild Type

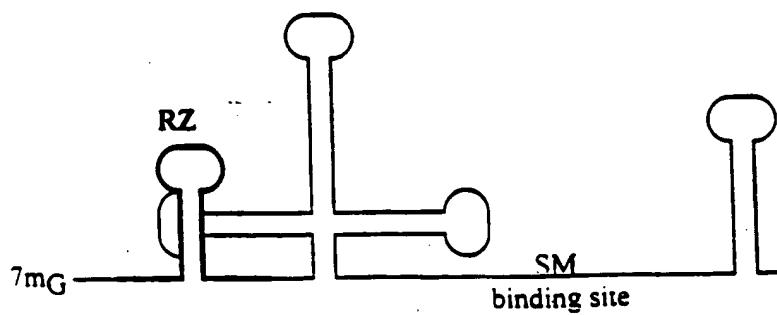
stable, exported to cytoplasm; binds SM, 2,2,7mG cap, goes back to nucleus (protein A1 binding)



stable, exported to cytoplasm (protein A1 binding)



stable, exported to cytoplasm



stable, exported to cytoplasm; binds SM, 2,2,7mG cap, goes back to nucleus (protein A1 binding)

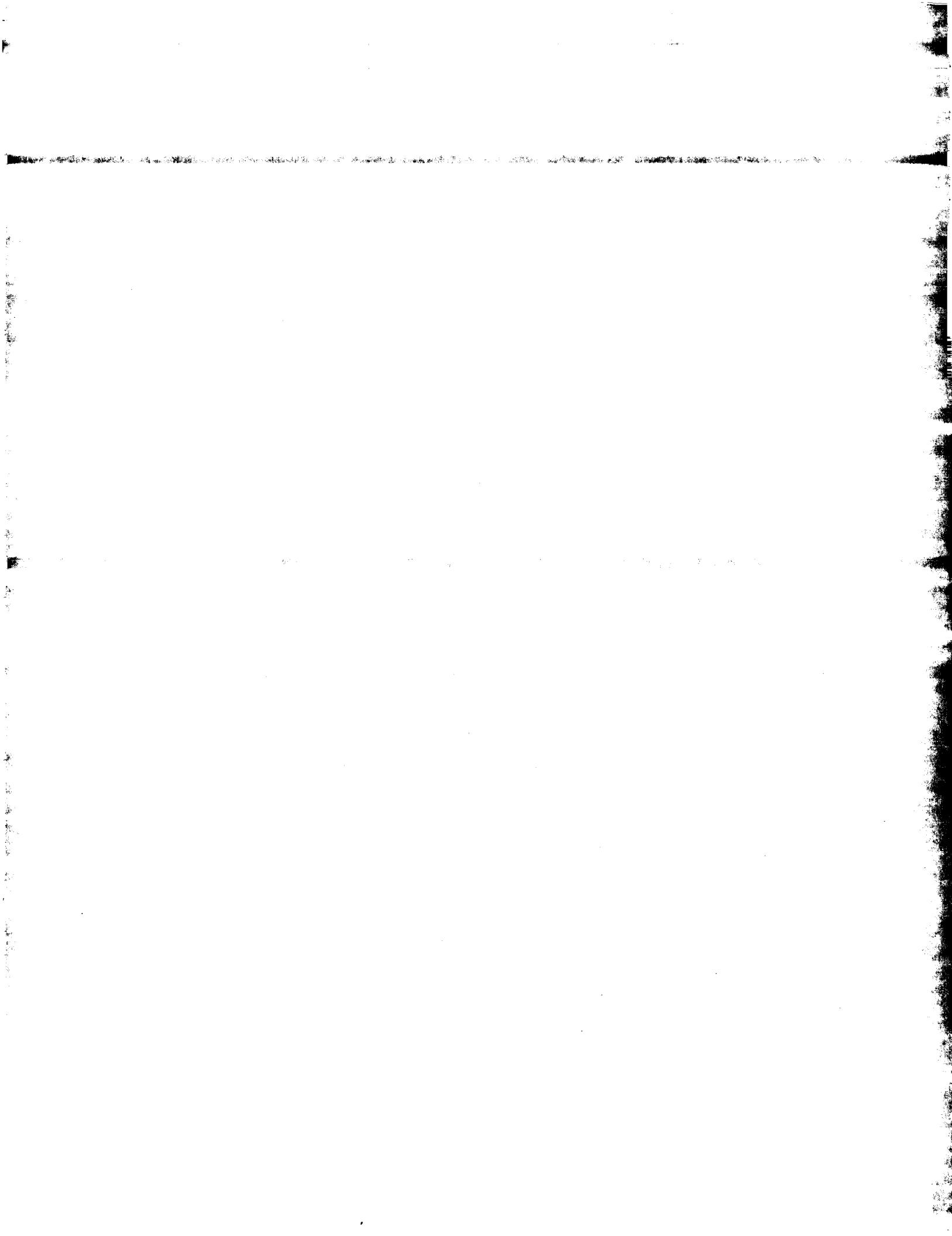
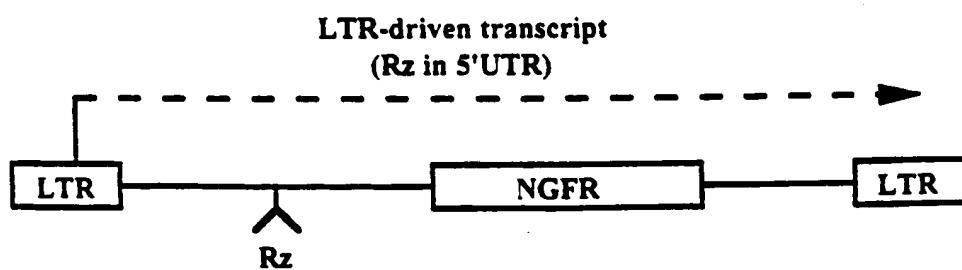
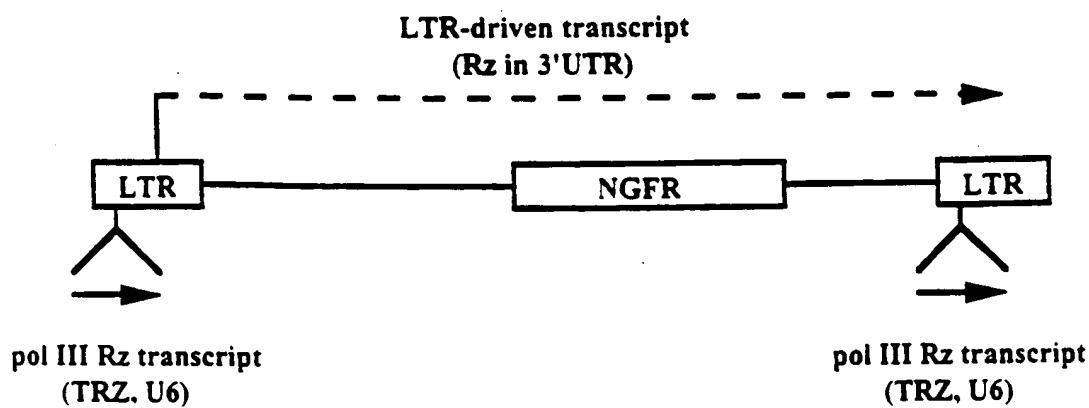
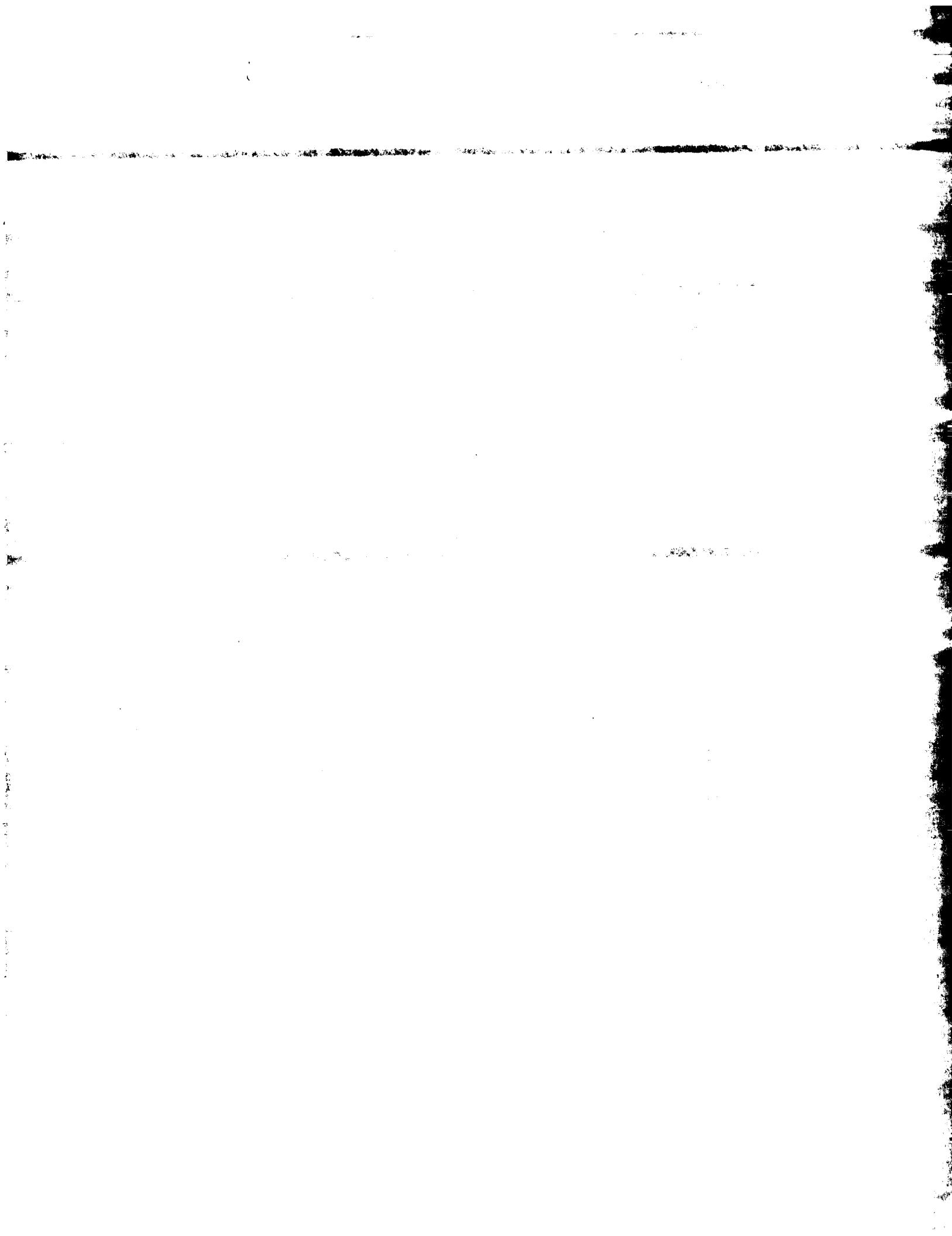
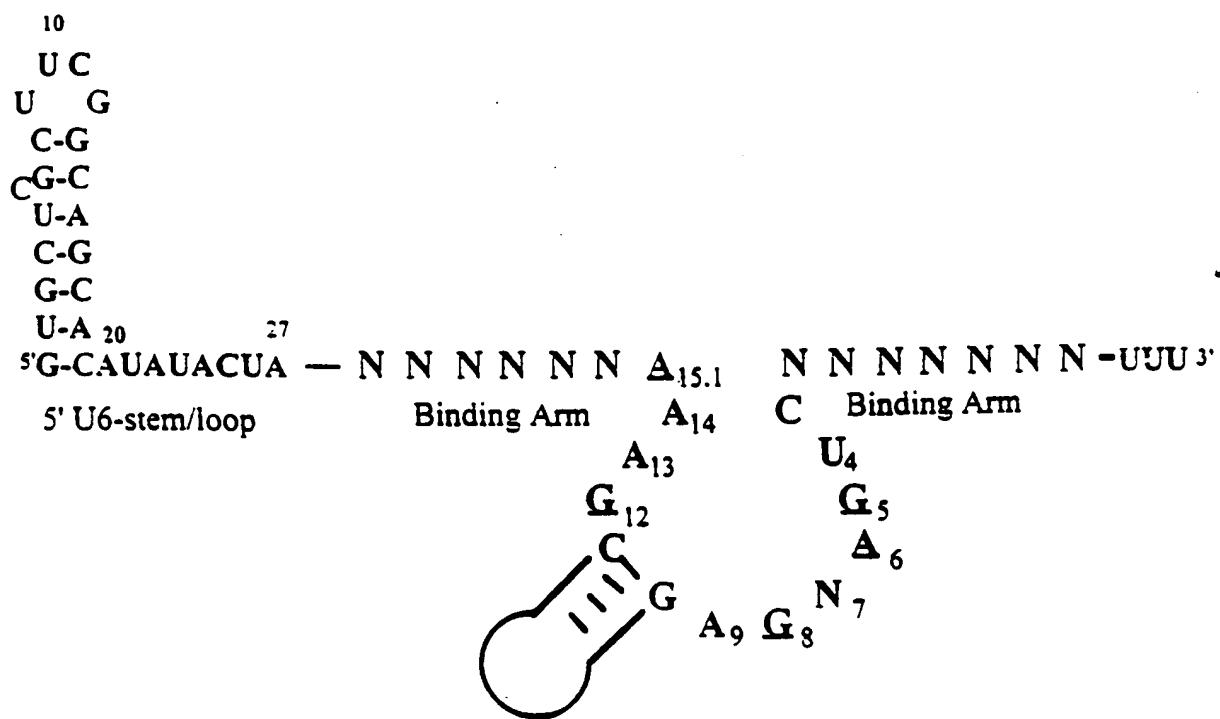
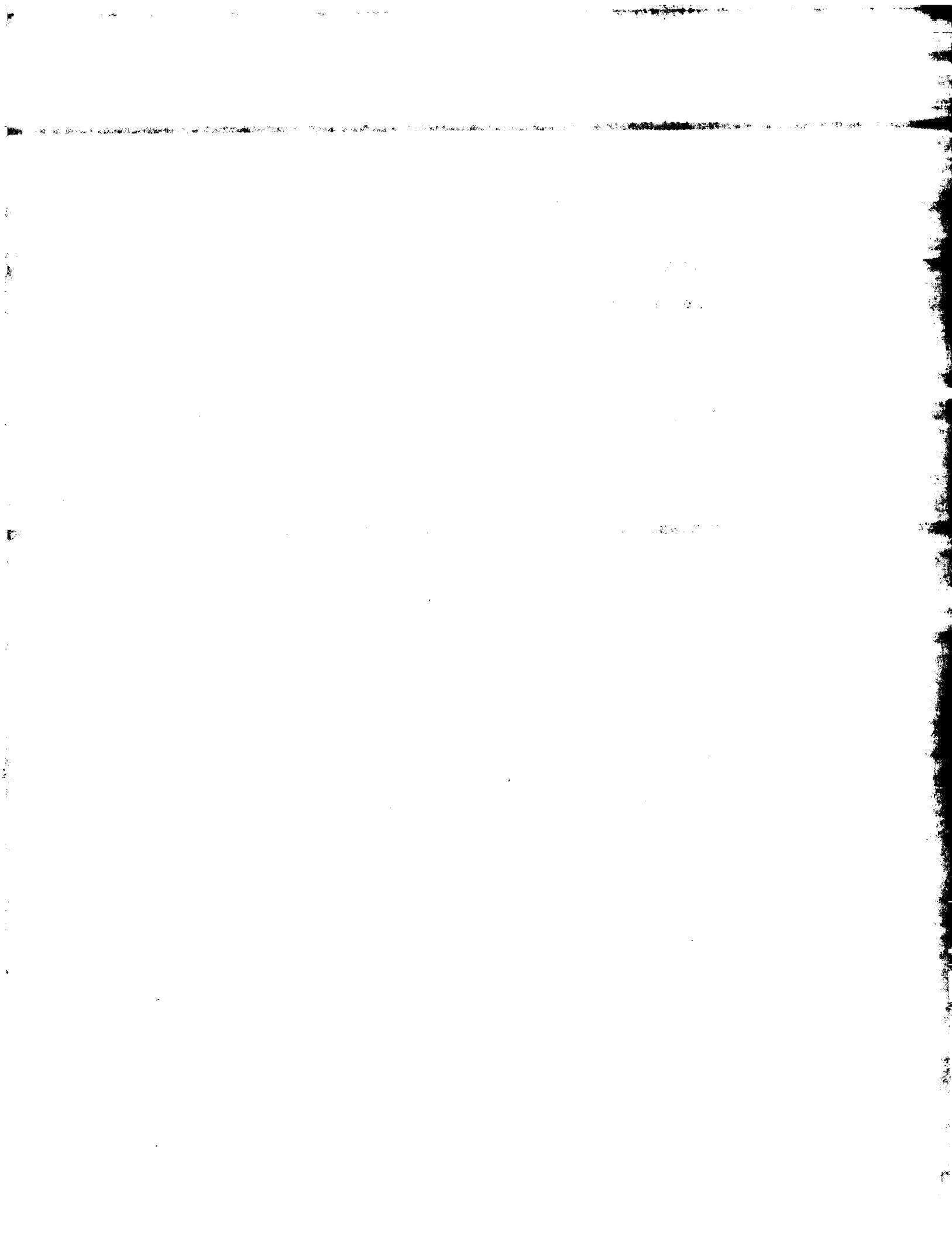


Figure 11C: Retroviral Vector Encoding Ribozymes

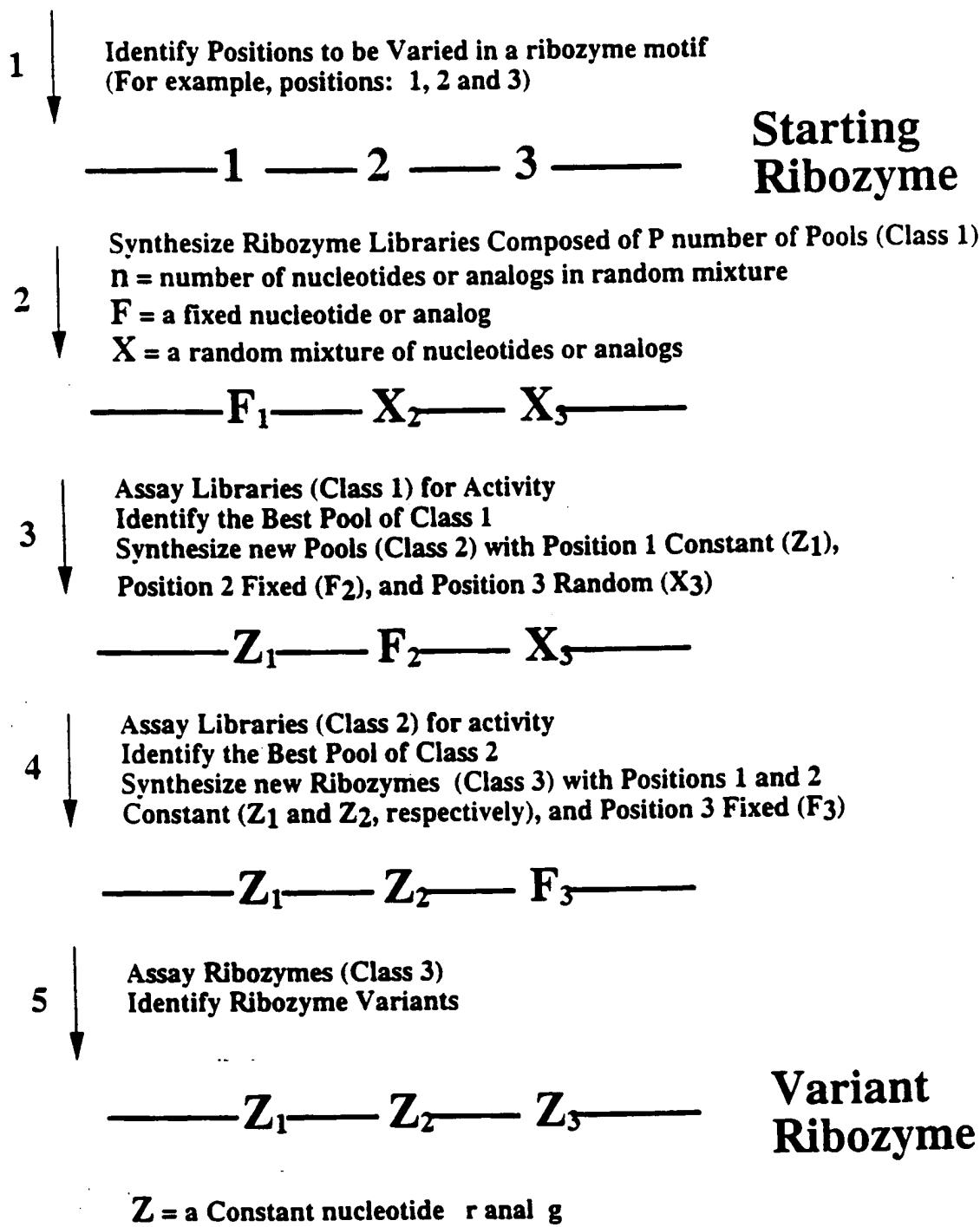




**Figure 11D: U6+27 Hammerhead Ribozyme Transcription Unit**

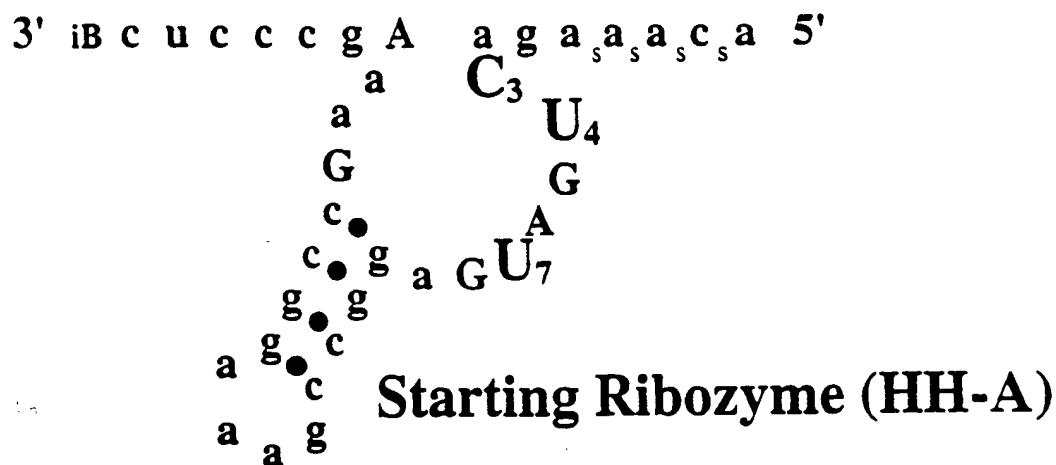


## Figure 12: A Combinatorial Approach to the Screening of Ribozyme Variants





### Figure 13: Starting Ribozyme Motif for Variation (Hammerhead Motif/HH Motif)

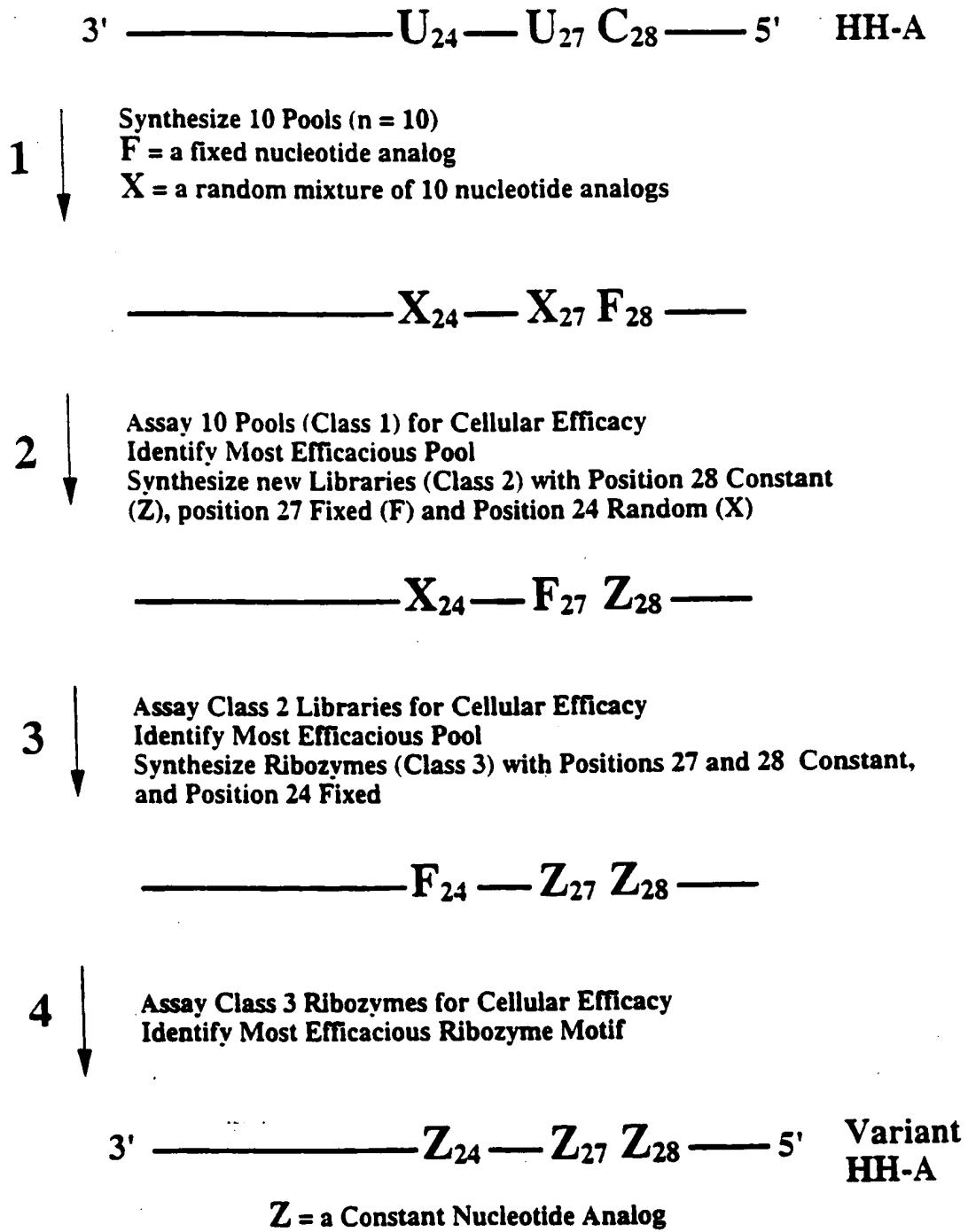


↓ Positions 3, 4 and 7 are being varied in one example





## Figure 14: Screening for Variants of HH-A Ribozyme





**Figure 15: Examples of Nucleotide Analogs (n) Used in Library (Class 1 Pool) Construction**

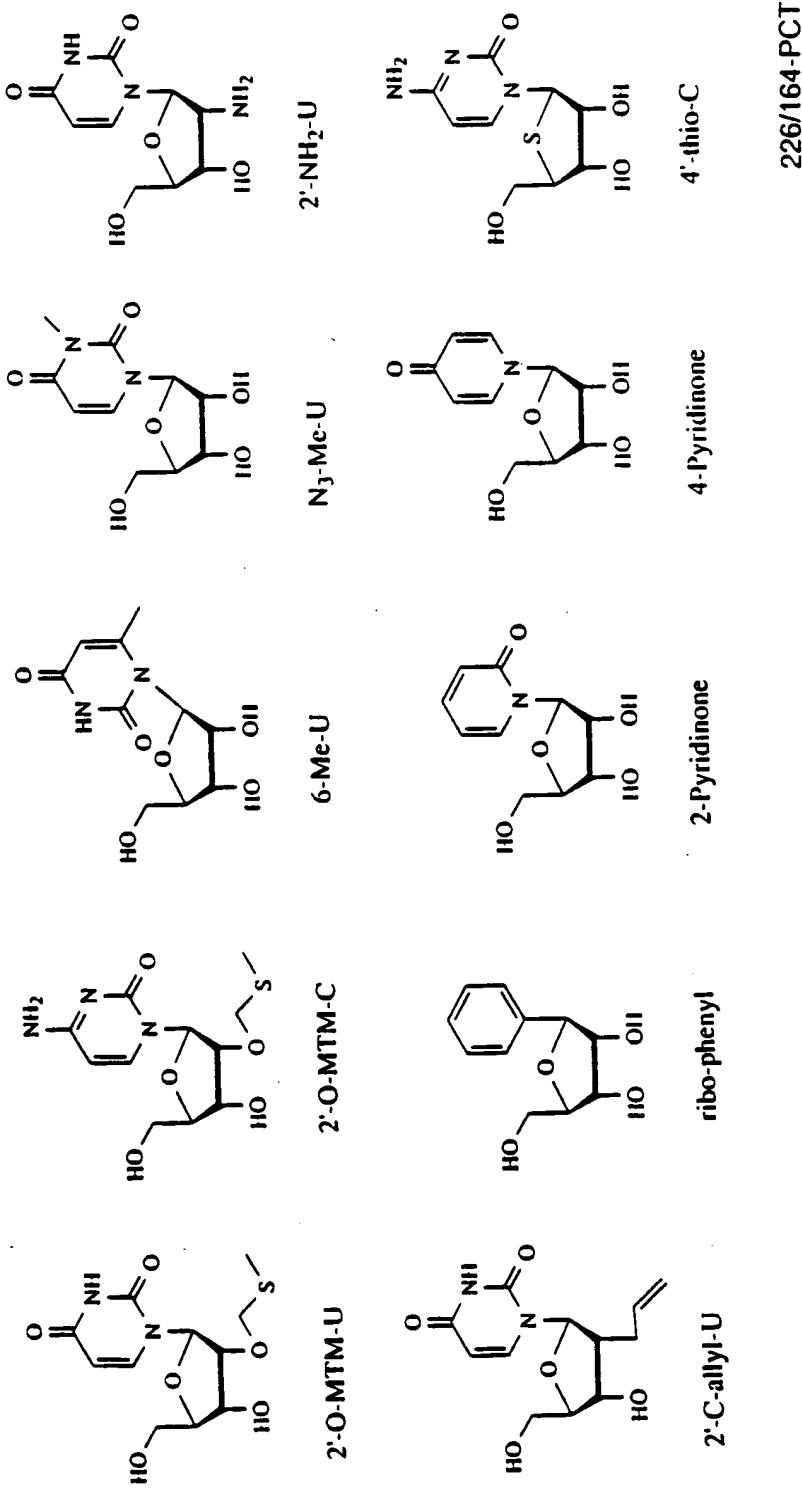
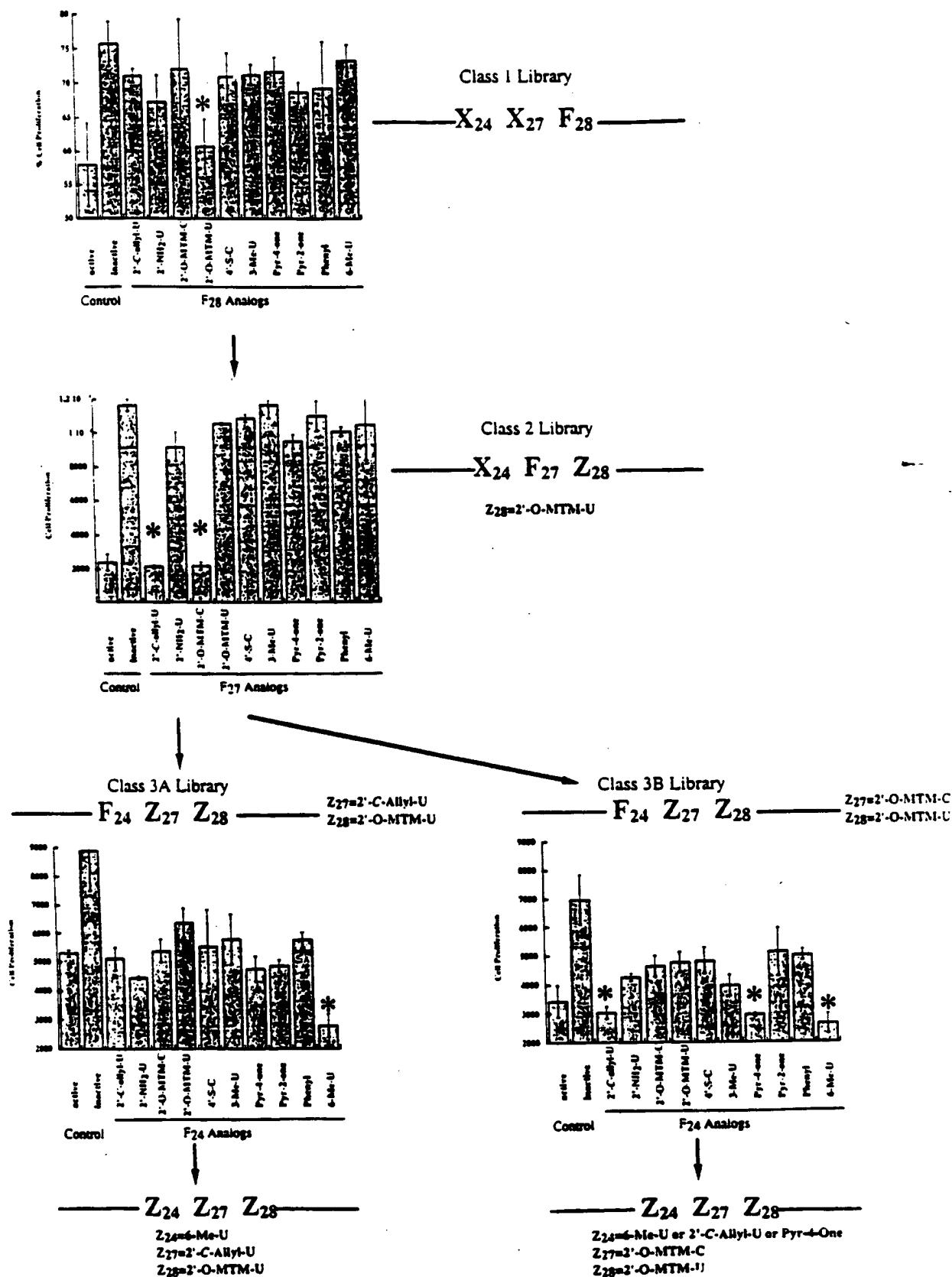




Figure 16: Activity of HH-A Variant Ribozymes



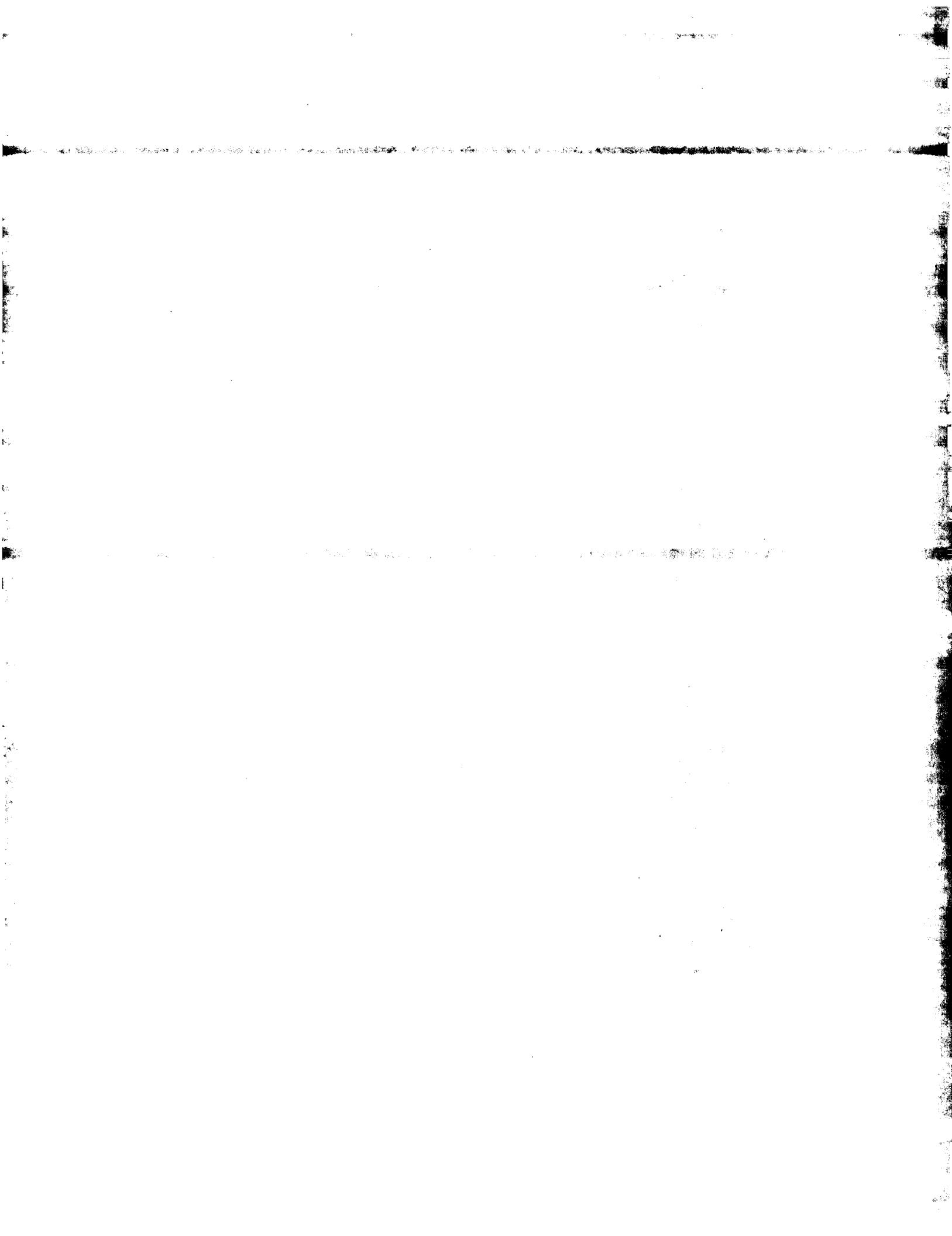
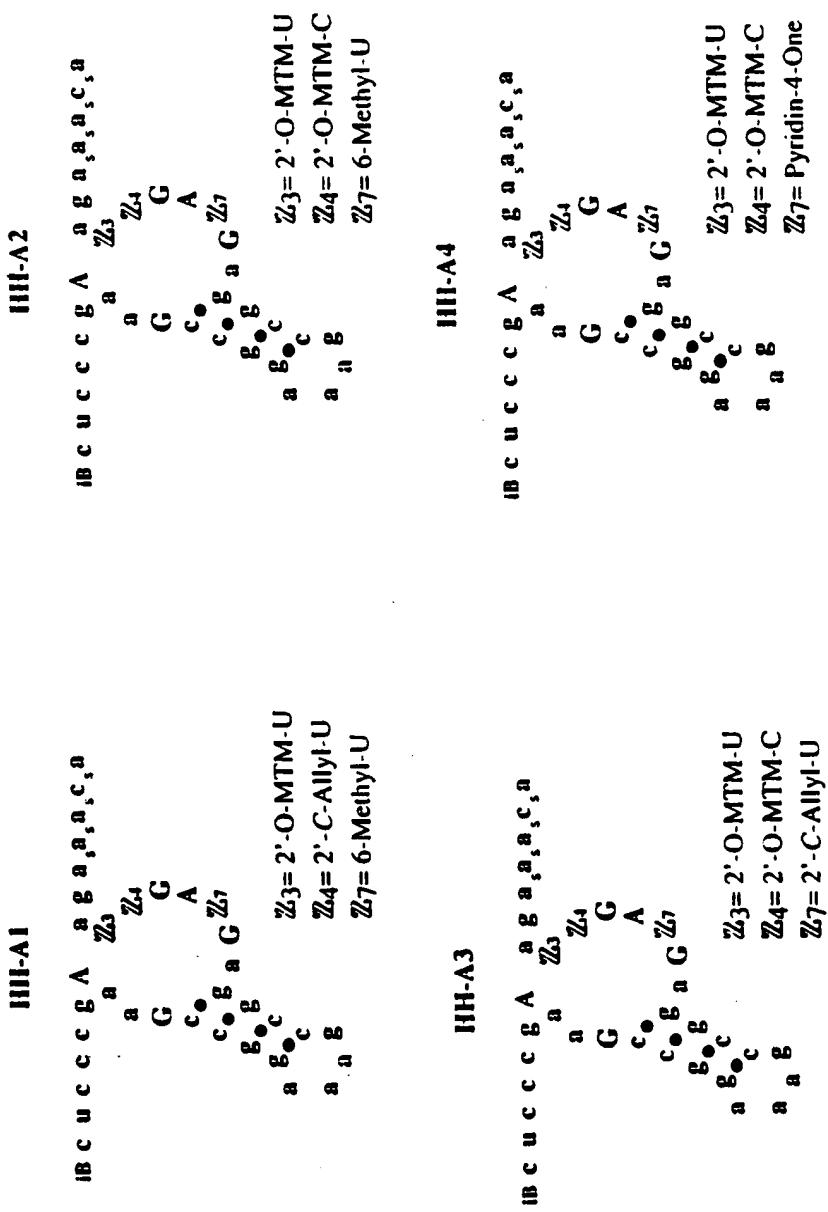
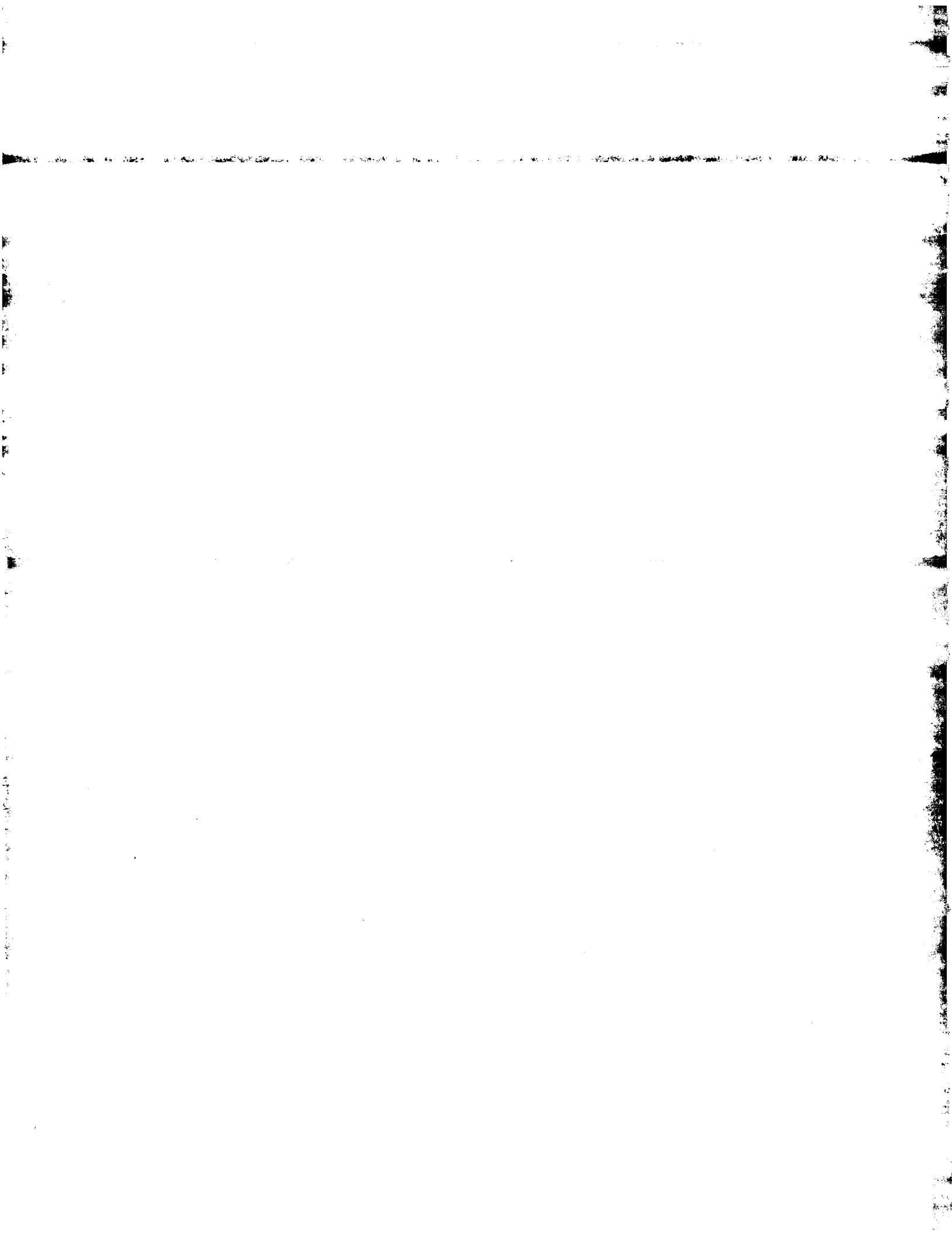
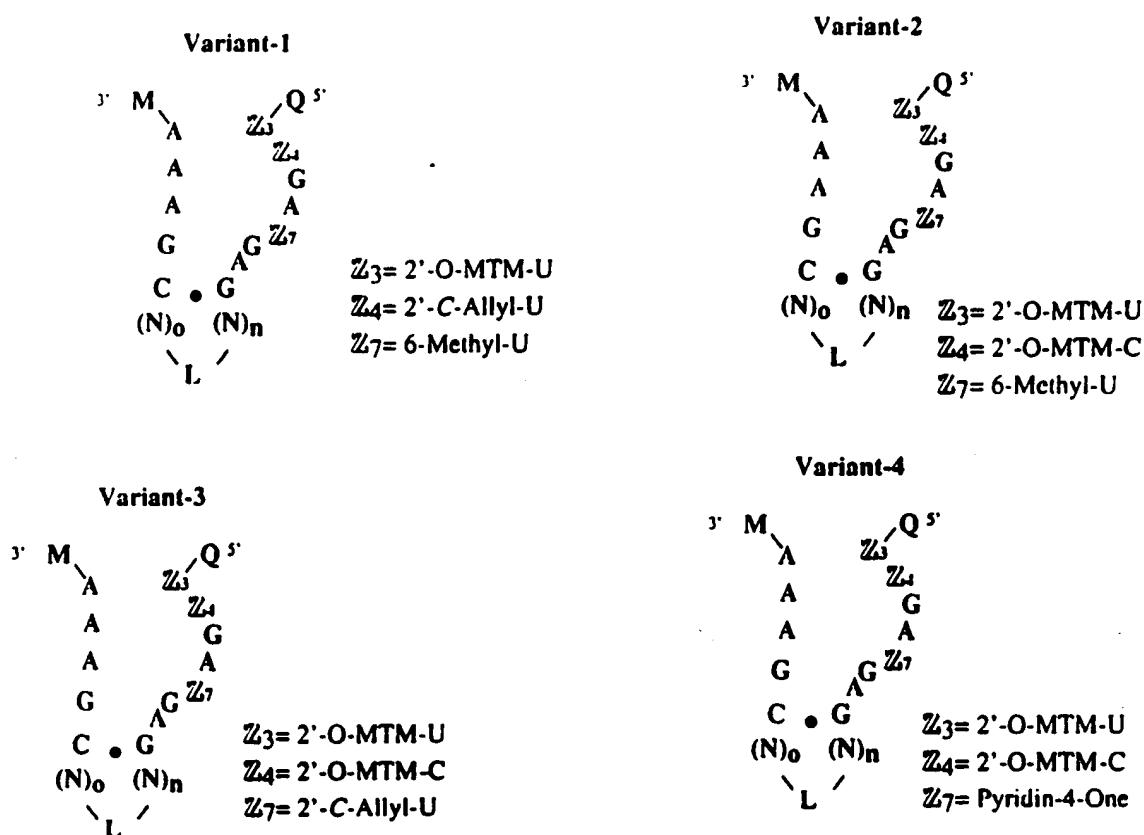


Figure 17A: III-A Variants





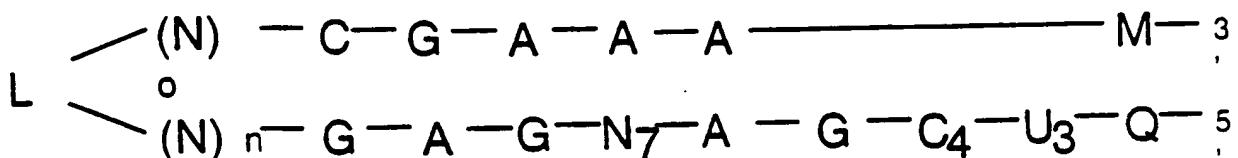
**Figure 17B: Novel Ribozyme Variants**



N is independently a nucleotide or a non-nucleotide linker, which may be same or different;  
 M and Q are independently oligonucleotides of length sufficient to stably interact (e.g., by forming hydrogen bonds with complementary nucleotides in the target) with a target nucleic acid molecule (the target can be an RNA, DNA or RNA/DNA mixed polymers);  
 L and R are integers greater than or equal to 1 and preferably less than about 100, wherein if (N)<sub>1</sub> and (N)<sub>2</sub> are nucleotides, (N)<sub>1</sub> and (N)<sub>2</sub> are optimally able to interact by hydrogen bond interaction;  
 L is a linker which may be present or absent (i.e., the molecule is assembled from two separate nucleotides), but when present, is a nucleotide and/or a non-nucleotide linker, which may be a single-stranded and/or double-stranded region; and  
 — represents a chemical linkage (e.g., a phosphate ester linkage, amide linkage or others known in the art).  
 A, C, U and G represent adenosine, cytidine, uridine and guanosine nucleotides, respectively, which may be modified or unmodified.



**Figure 18: Formula for a novel Ribozyme Motif**



$\text{N}$  = independently a nucleotide or a non-nucleotide linker, which may be same or different;

$\text{M}$  and  $\text{Q}$  are independently oligonucleotides of length sufficient to stably interact (e.g., by forming hydrogen bonds with complementary nucleotides in the target) with a target nucleic acid molecule (the target can be an RNA, DNA or RNA/DNA mixed polymers);  $\text{o}$  and  $n$  are integers greater than or equal to 1 and preferably less than about 100, wherein if  $(\text{N})\text{o}$  and  $(\text{N})\text{n}$  are nucleotides,  $(\text{N})\text{o}$  and  $(\text{N})\text{n}$  are optionally able to interact by hydrogen bond interaction;

$\text{L}$  is a linker which may be present or absent (i.e., the molecule is assembled from two separate molecules), but when present, is a nucleotide and/or a non-nucleotide linker, which may be a single-stranded and/or double-stranded region; and

— represents a chemical linkage (e.g. a phosphate ester linkage, amide linkage or others known in the art).

$\text{A}$ ,  $\text{C}$ ,  $\text{U}$  and  $\text{G}$  represent adenosine, cytidine, uridine and guanosine nucleotides, respectively, which may be modified or unmodified.

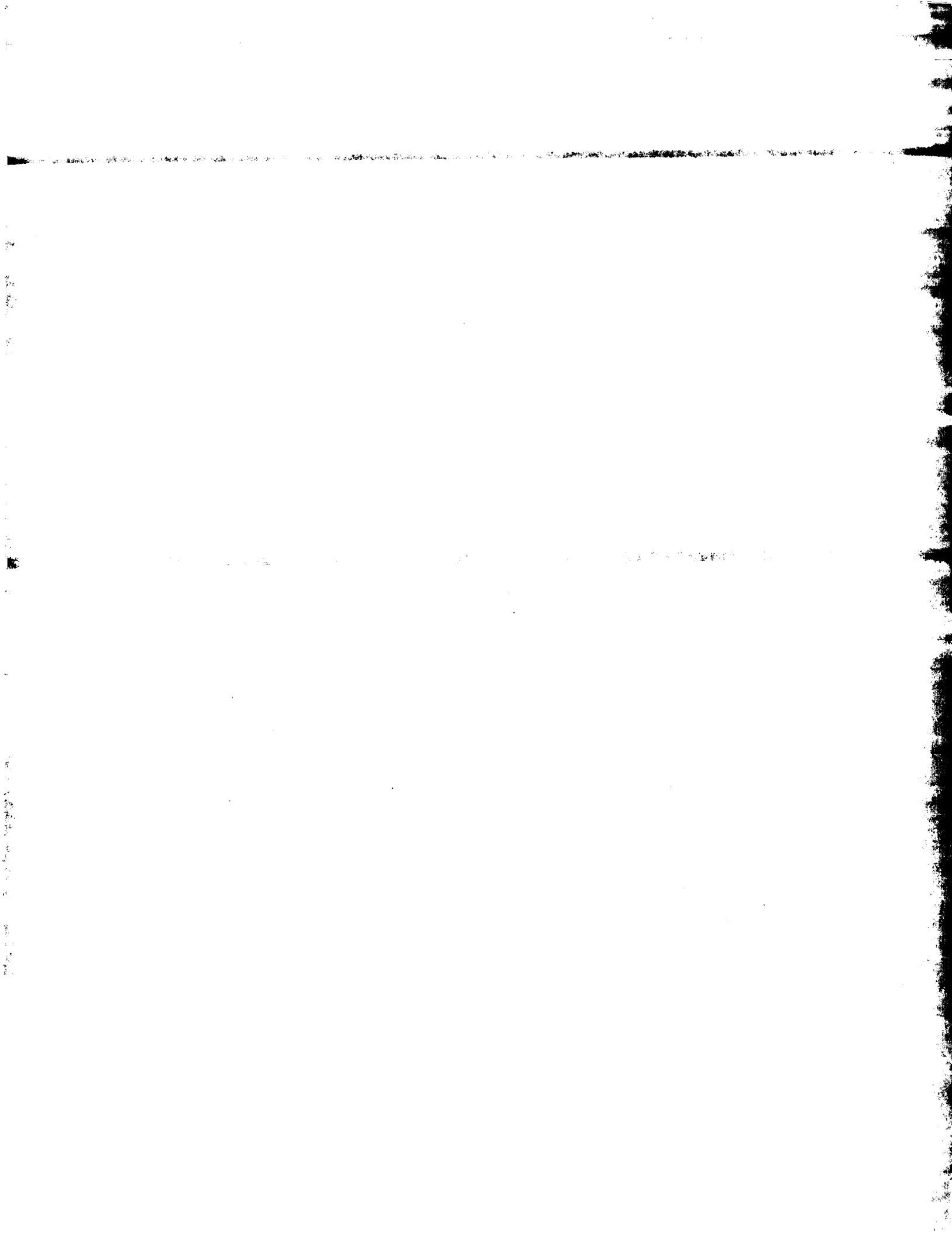
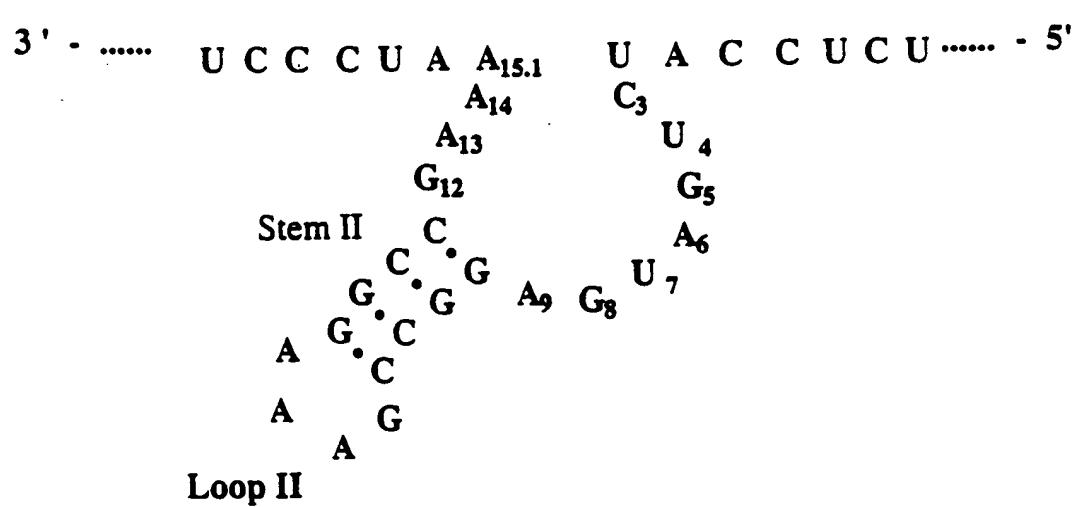
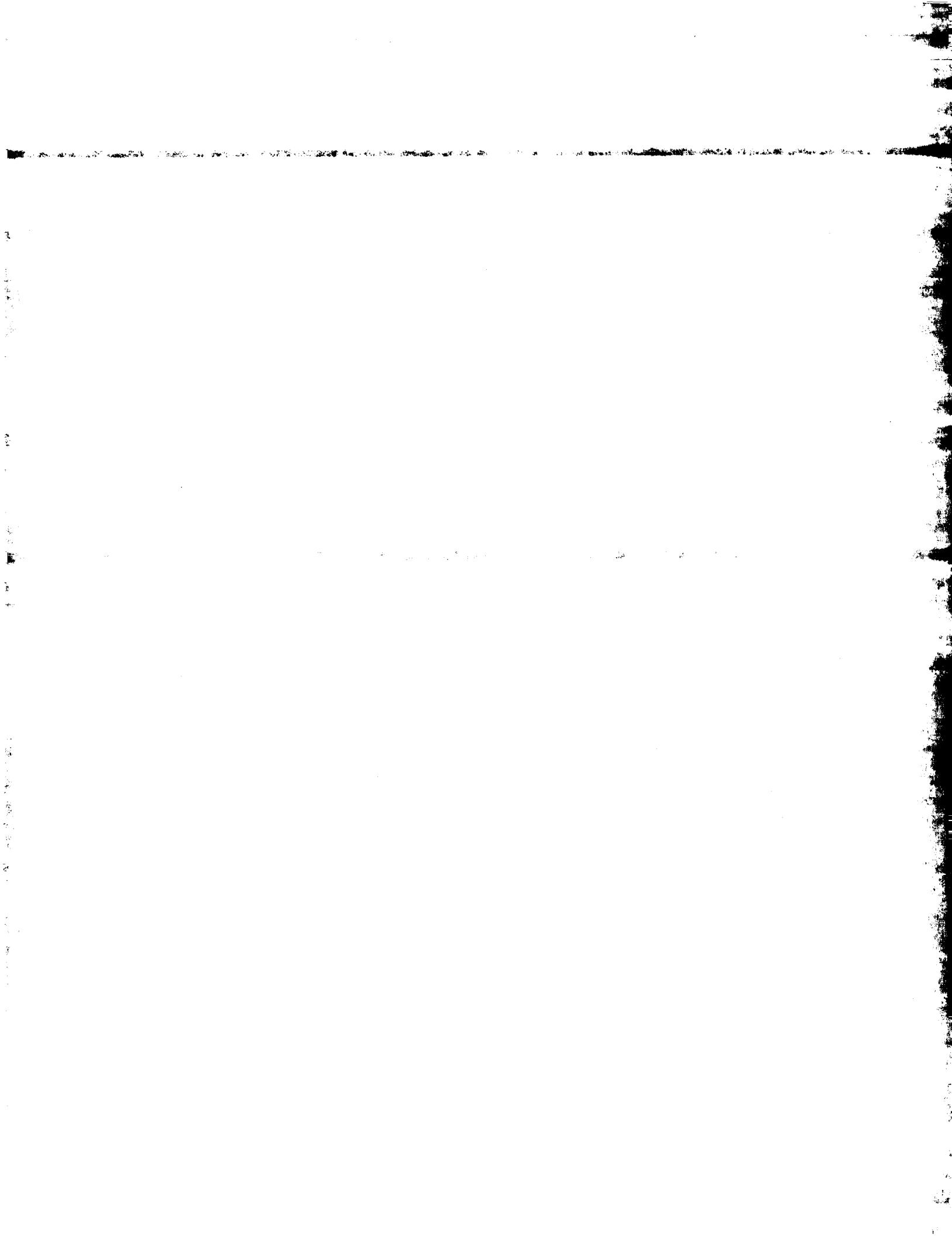


Figure 19: Starting Ribozyme motif for Loop Variation



Hammerhead Ribozyme B (HH-B)



## Figure 20: Screening for Loop-II Variants of Hammerhead Ribozyme B (HH-B)

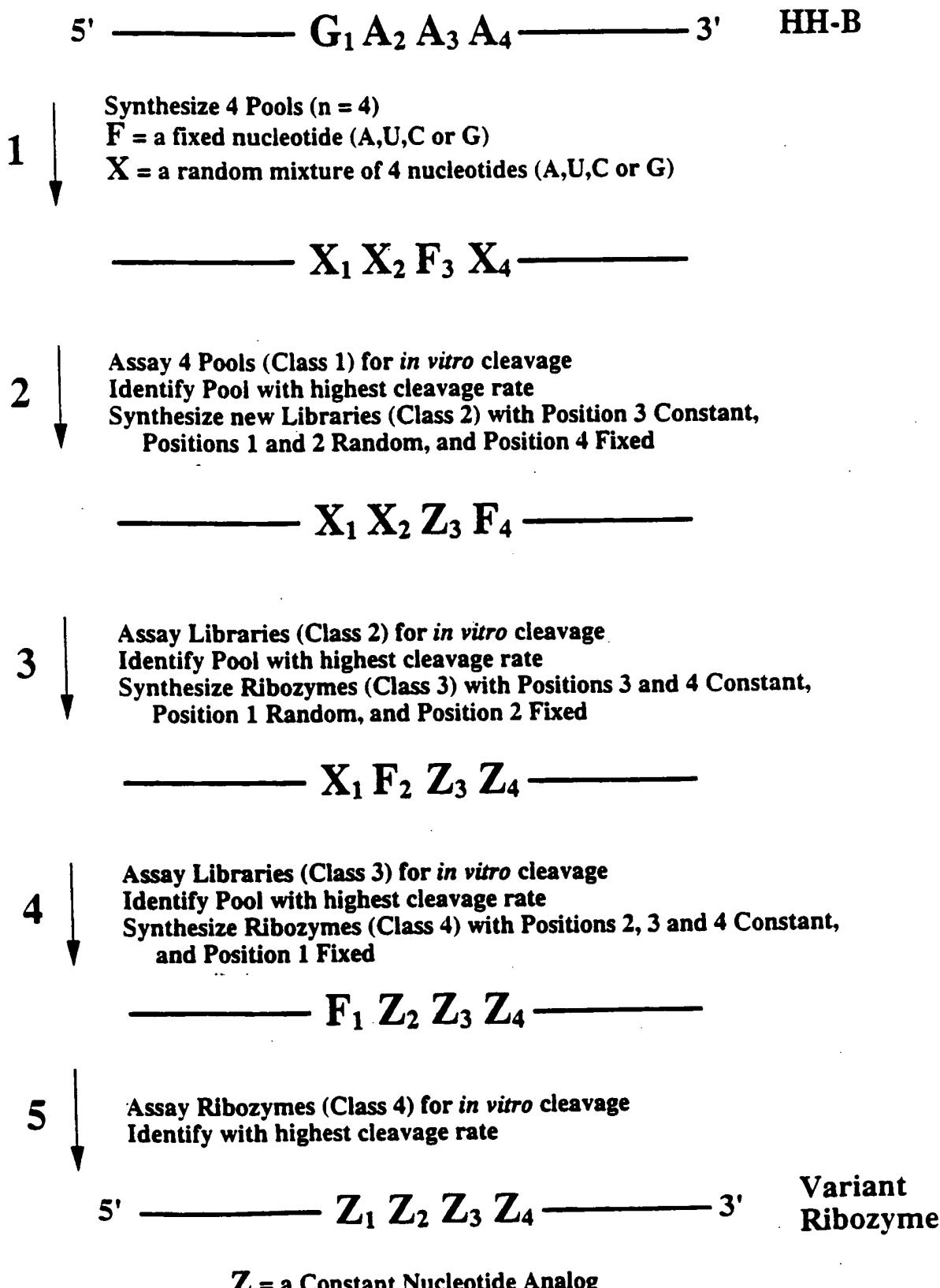
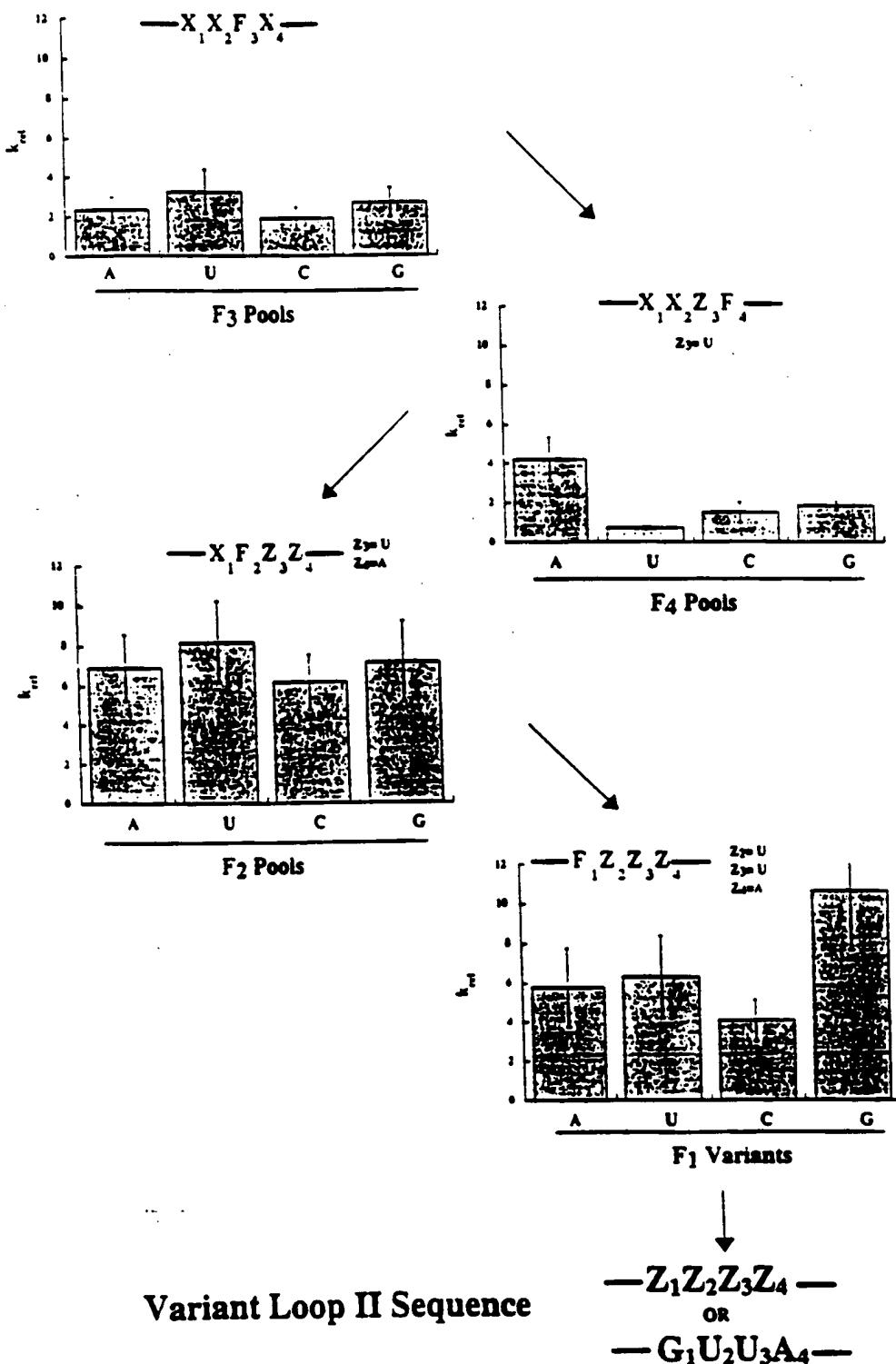




Figure 21: Activity of HH-B Variant Ribozymes



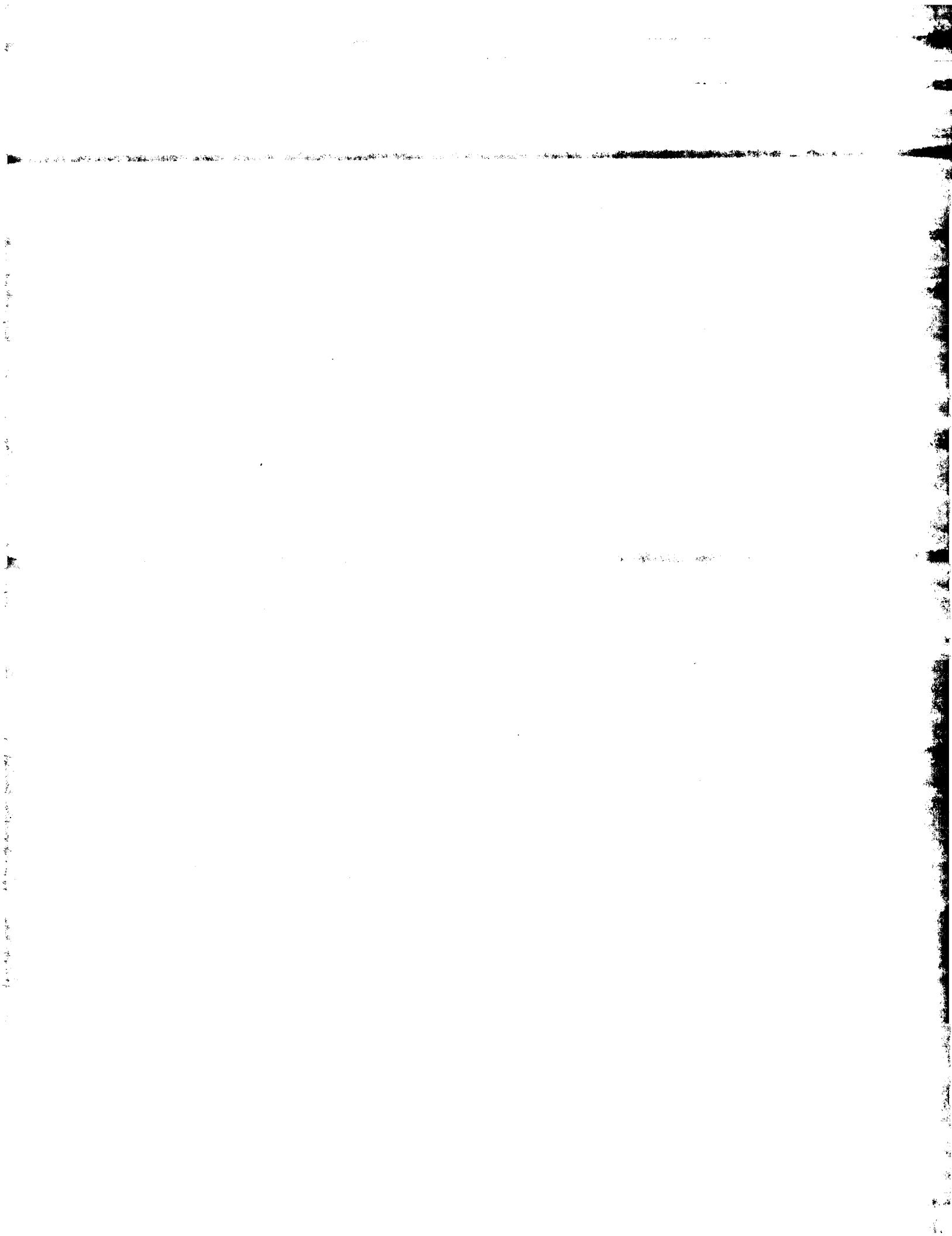
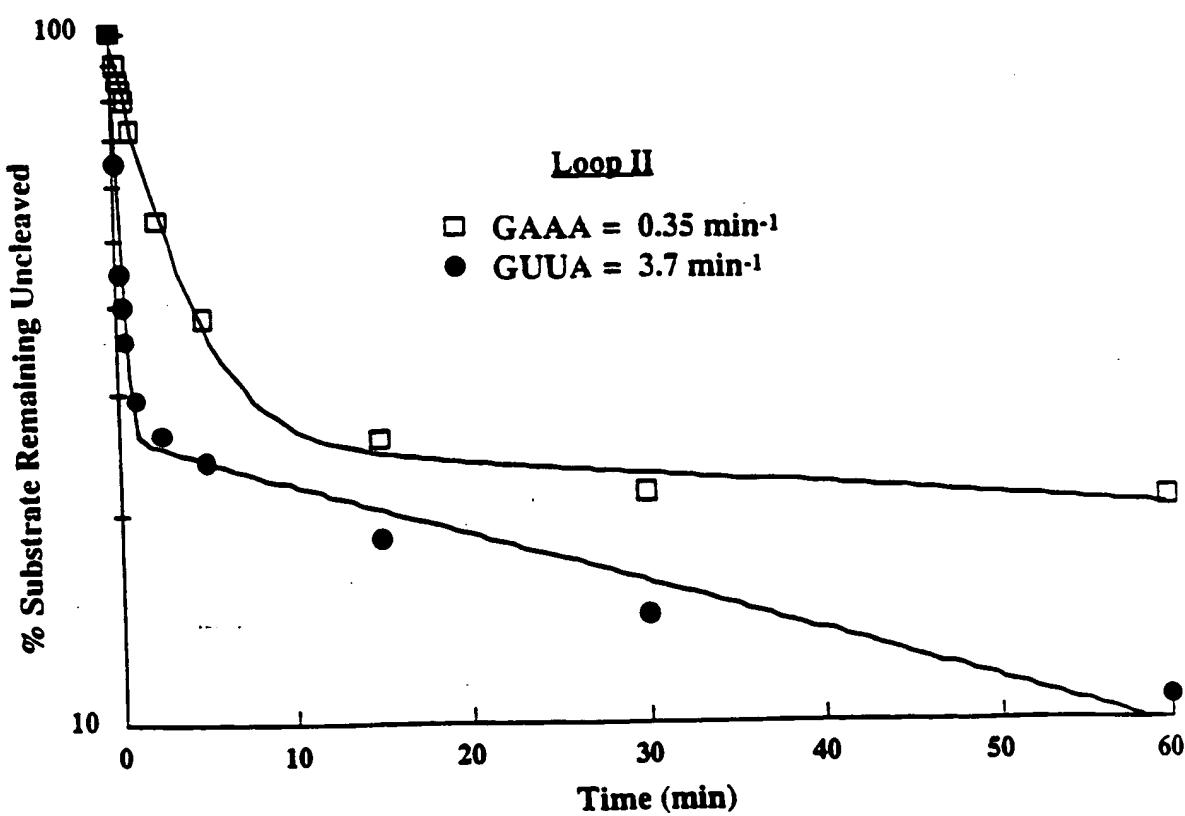
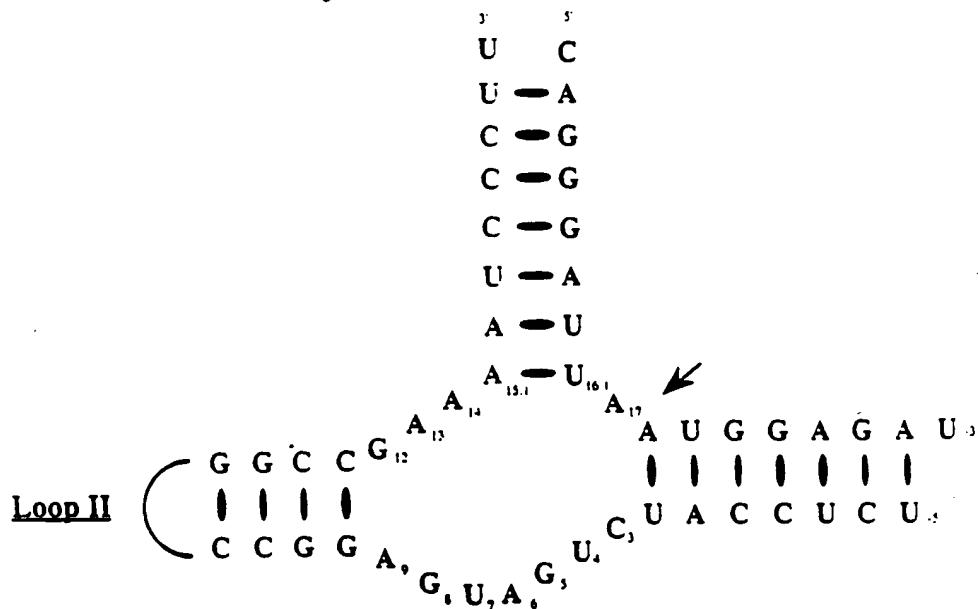


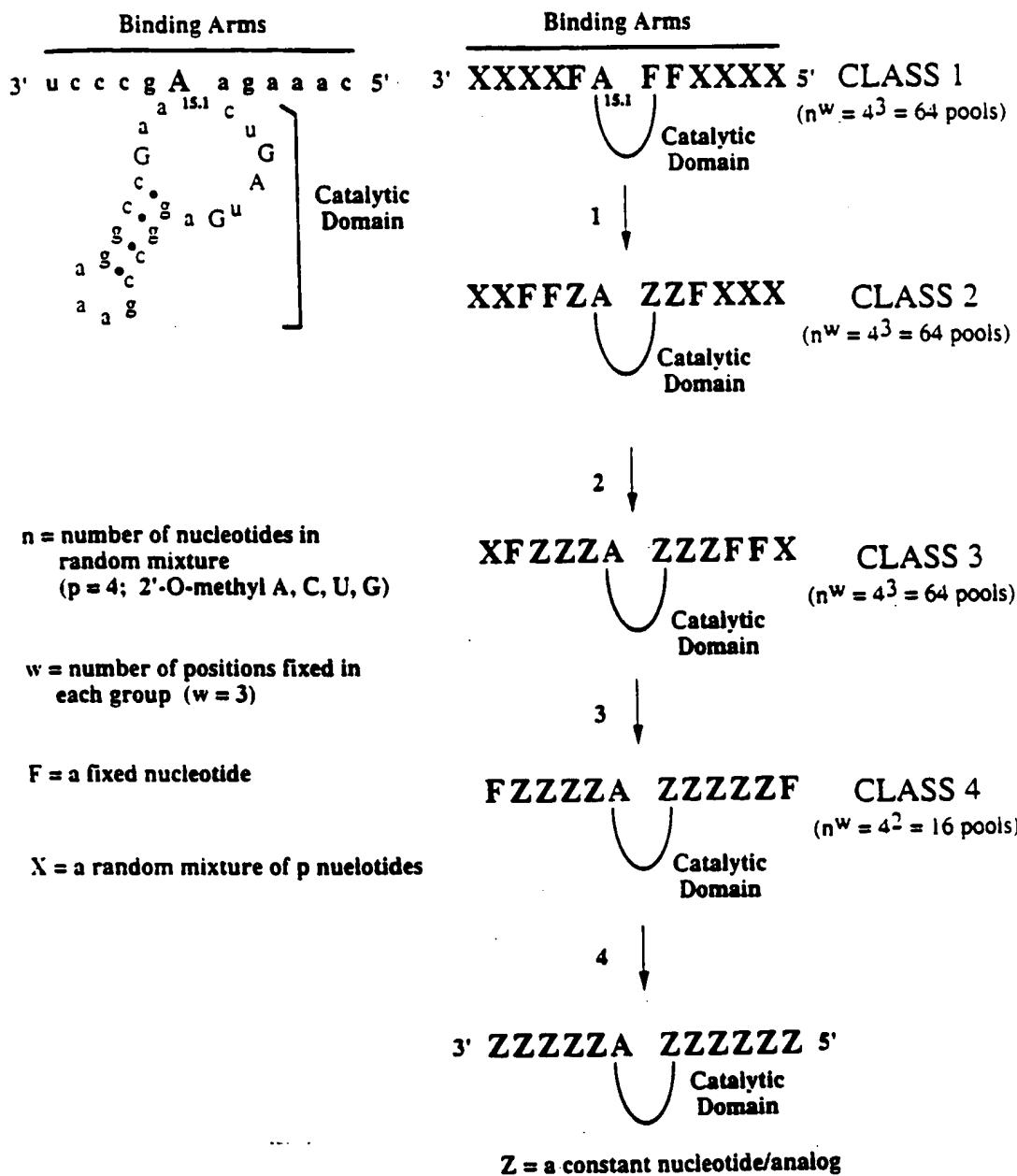
Figure 22:RNA Cleavage by Loop II Variant HH-B Ribozyme





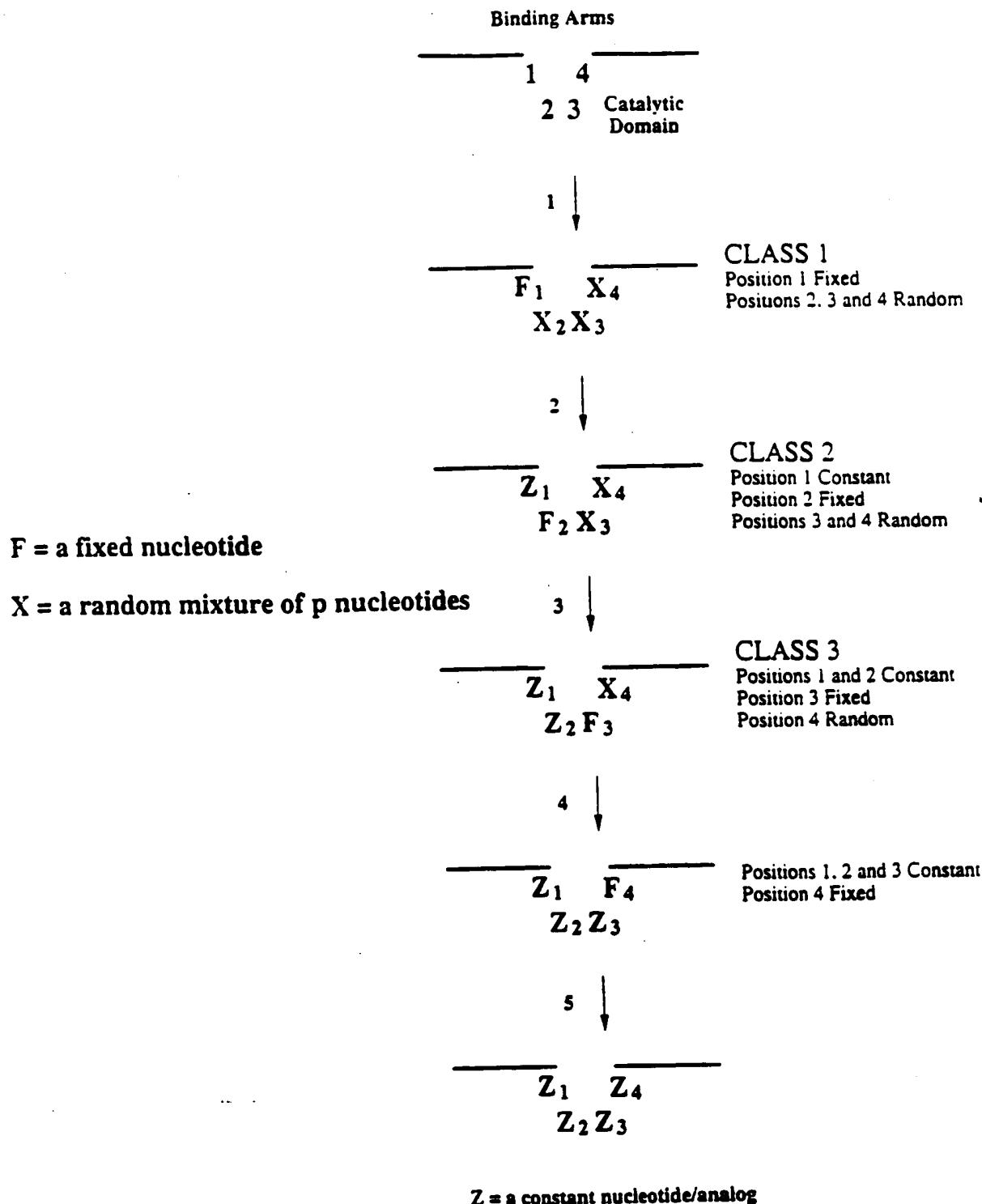
# Figure 23: Screening for Ribozyme Binding Arm Sequence Variants-Target Discovery

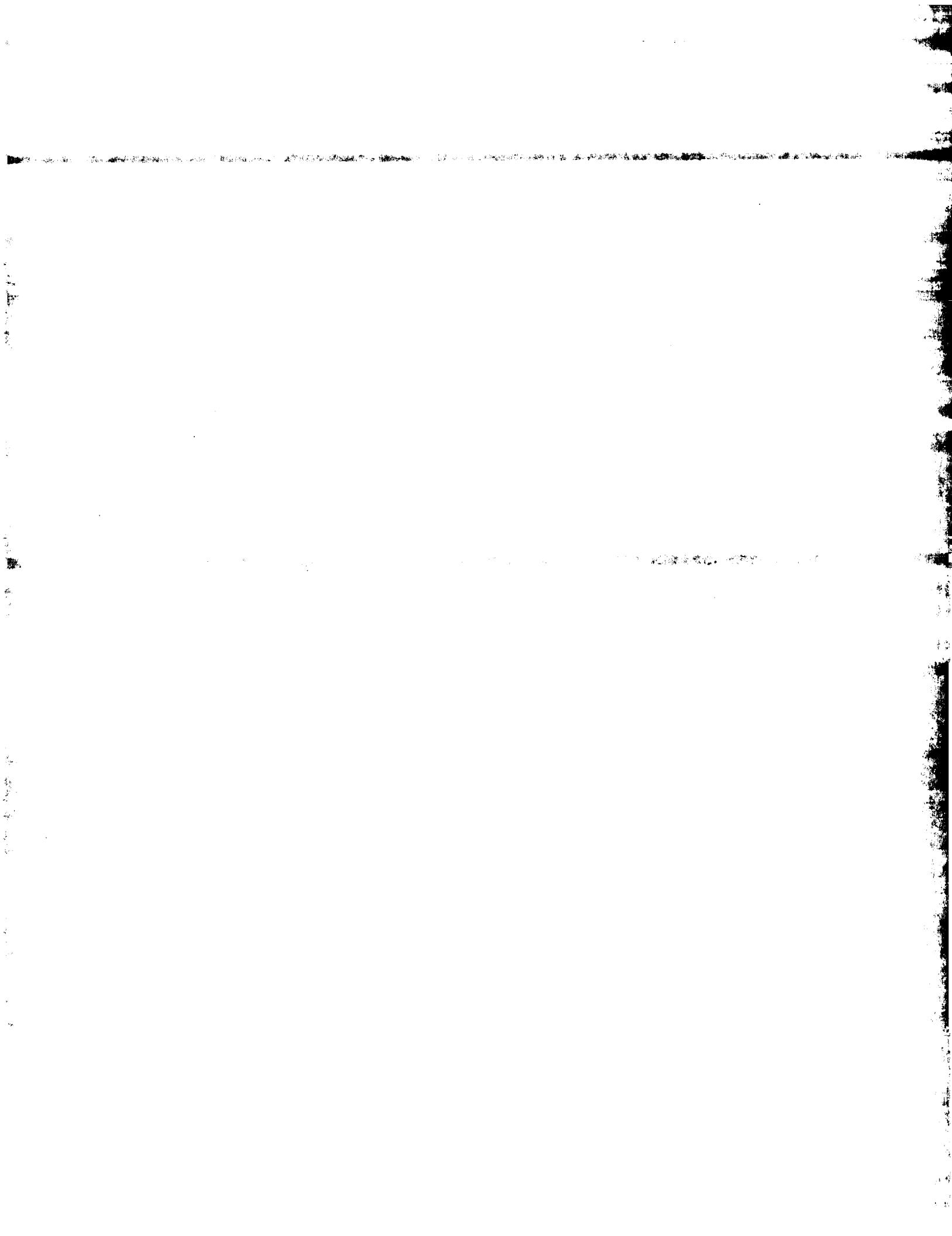
## Hammerhead Ribozyme Model System





## Figure 24: Screening for Ribozyme Catalytic Domains

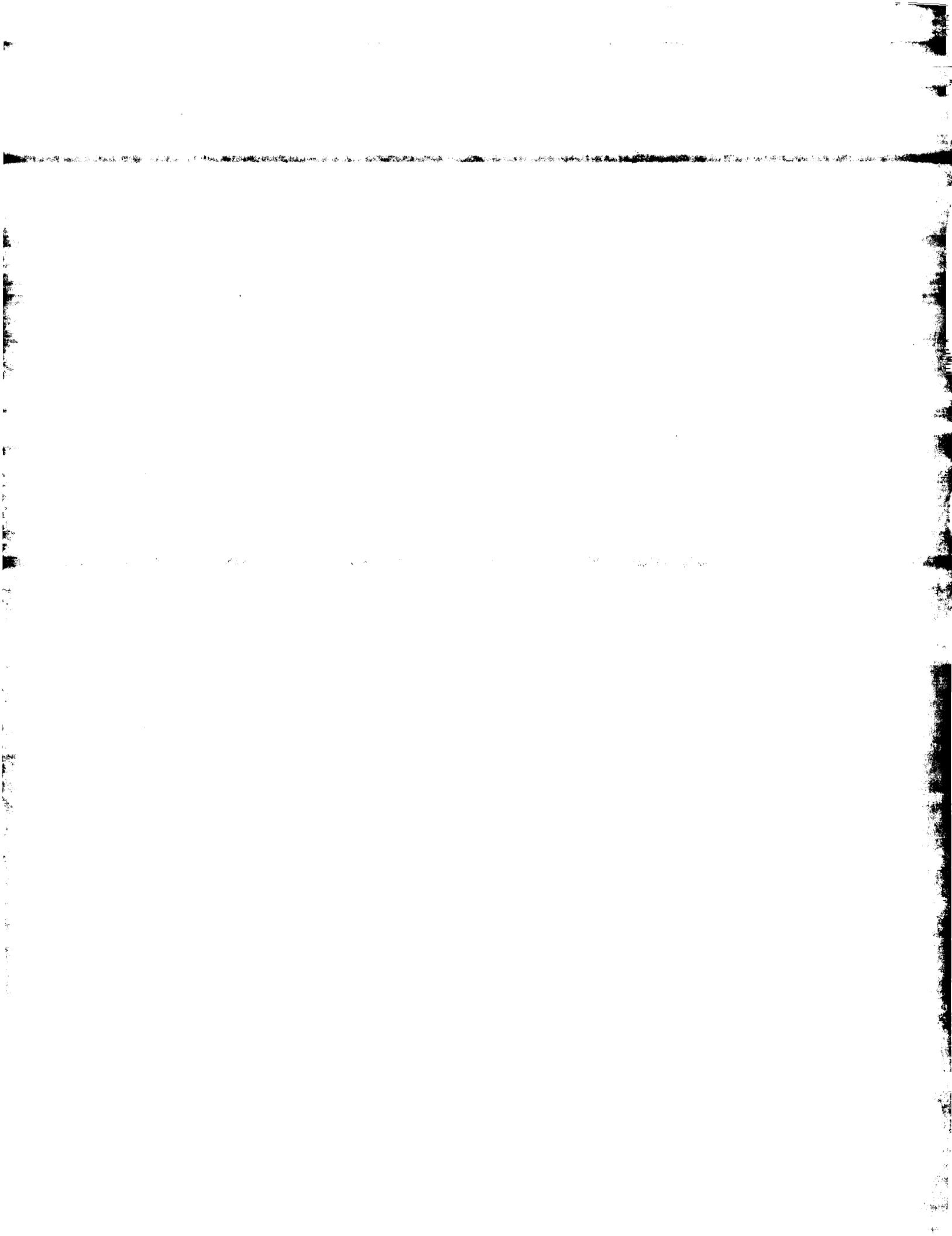




**Figure 25: Accessible Sites Within a Bcl-2 Transcript Determined Using Combinatorial *In Vitro* Screen.**

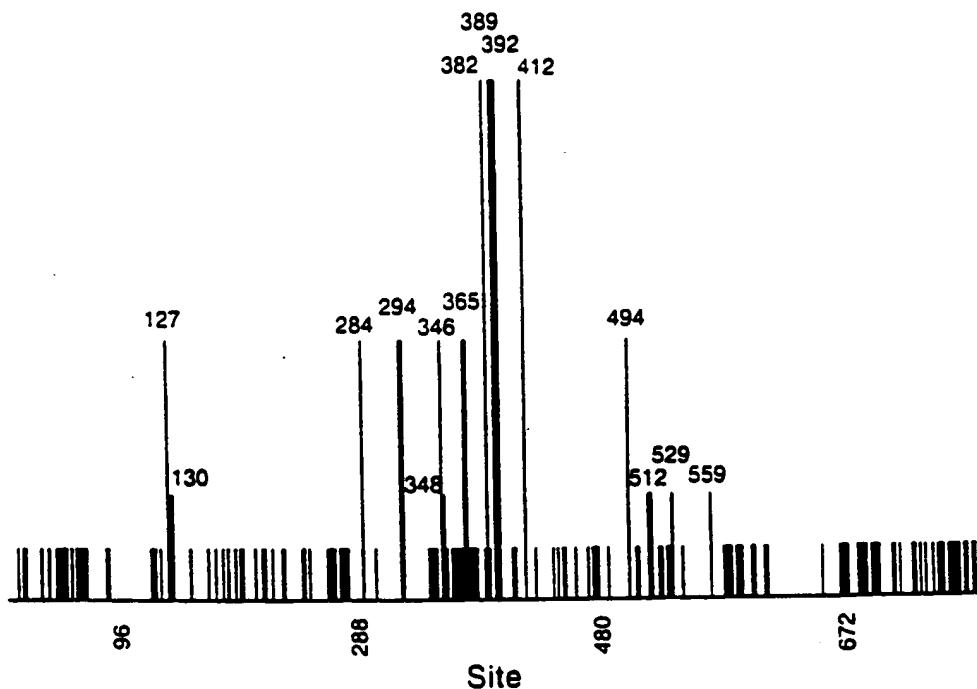
<u>Seq. ID No.</u>	<u>Sequence of Site</u>	<u>Site</u>	<u>Est. Size</u>
1	TTGCTTT TCCCTCT	78	80
2	GTTGCTT TTCCCTC	77	80
3	GTGCGCTA TCTGAG	795	800
4	GCTCCTC TAGACT	25	30
5	CGCCCTT CACCCG	462	480
6	AGCTCTT CAGGGA	504	480
7	TCCCTCTA GACTCG	32	30
8	CTGAGTA CCTGAA	630	650
9	TTGAGTT CGGIGG	549	550
10	TGAAGTA CATCCA	144	160
11	TGTGGTC CACCTG	370	370
12	CCCCATC CAGCCG	266	264
13	CTGGATC CAGGAT	658	680

Bcl-2 transcript is 975 nts. in length. Transcript was generated from a cDNA clone. 101 potential ribozyme sites (NUH rule); combinatorial screen identified 13 accessible sites *in vitro*.



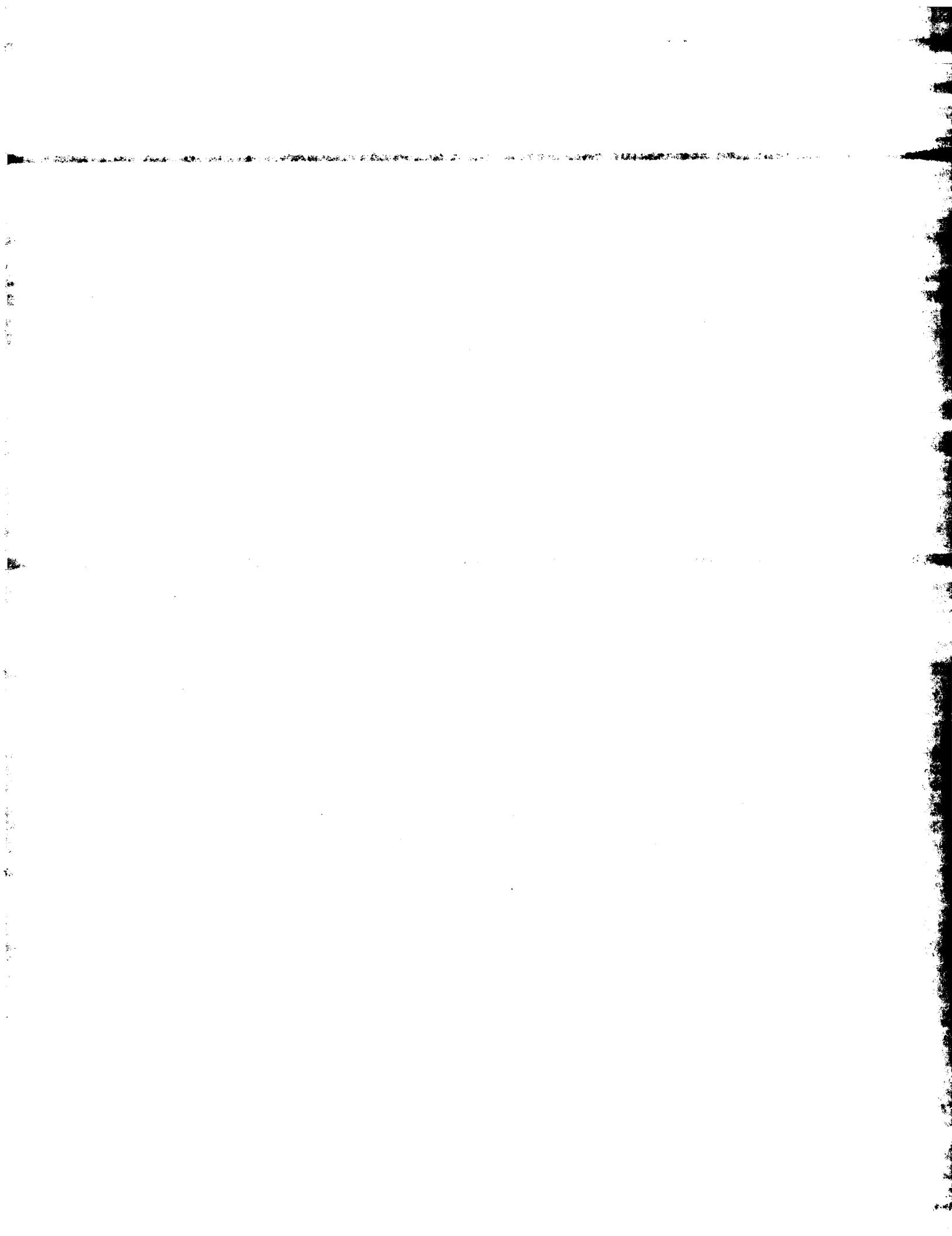
**Fig.26: Accessible Sites Within a K-ras Transcript Determined Using Combinatorial *In Vitro* Screen.**

### Kras Combinatorial In Vitro Screen



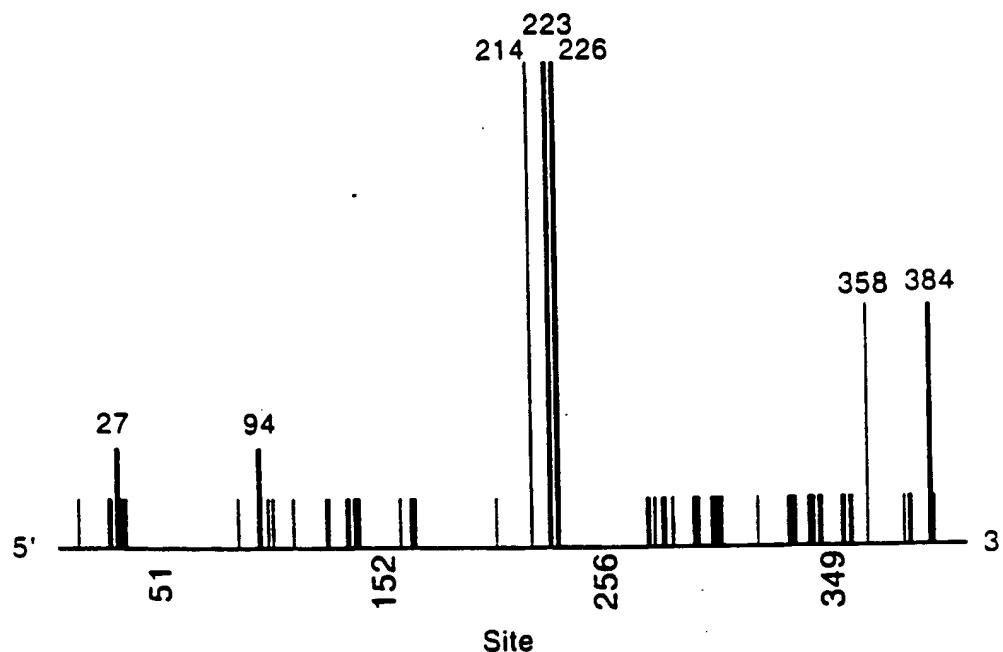
<u>Seq.ID No.</u>	<u>Sequence of Site</u>	<u>Site</u>	<u>Est. Size</u>
14	CAGGCTC AGGAGT	494	500
15	AATACTA AATCAT	365	370
16	TIGIGIA TTIGCC	346	350
17	AGGAGT ACAGTGC	294	300
18	TGIGGTA GTTGA	127	120
19	GGTAGIT CGAGCT	130	130
20	GGGIGIT GACGAT	559	570
21	AGGAGIT ATGGGA	512	530
22	CCAGGTC AAGAGG	284	280
23	AAGAGTA AAGGAC	412	410
24	GTGTATT TGCCAT	348	360
25	AGATATT CACCAT	382	390
26	CATTATA GAGAAC	392	390
27	ATTCAIT GAGACC	529	540
28	CACCAIT ATAGAG	389	390

Kras transcript is 796 nts. in length. Transcript was generated from a cDNA clone (rat). 144 potential ribozyme sites (NUH rule); combinatorial screen identified 15 accessible sites *in vitro*.



**Fig. 27. Accessible Sites Within a Urokinase Plasminogen Activator Transcript Determined Using Combinatorial *In Vitro* Screening.**

### UPA Combinatorial In Vitro Screen

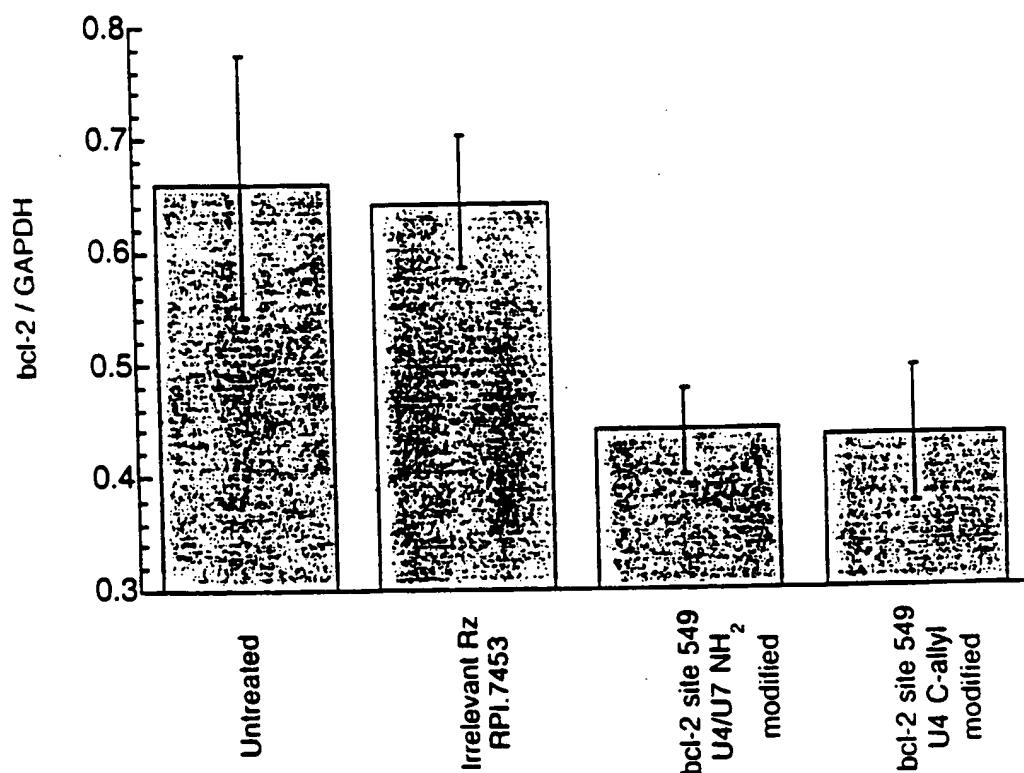


<u>Seq.ID No.</u>	<u>Sequence of Site</u>	<u>Site</u>	<u>Est. Size</u>
29	GTCACIT TTACCG	27	30
30	GGCGCTT GTCCAA	223	220
31	GGGCCTA AAGCCG	214	210
32	CACTGTC TTTCAG	94	100
33	GCTTGTGTC CAAGAG	226	230
34	GGCCATC TACAGG	385	400
35	CACCATC GAGAAC	358	370

UPA transcript is 400 nts. in length. Transcript was generated from a PCR generated fragment (contains a T7 promoter). 45 potential ribozyme sites (NUH rule); combinatorial screen identified 7 accessible sites *in vitro*.

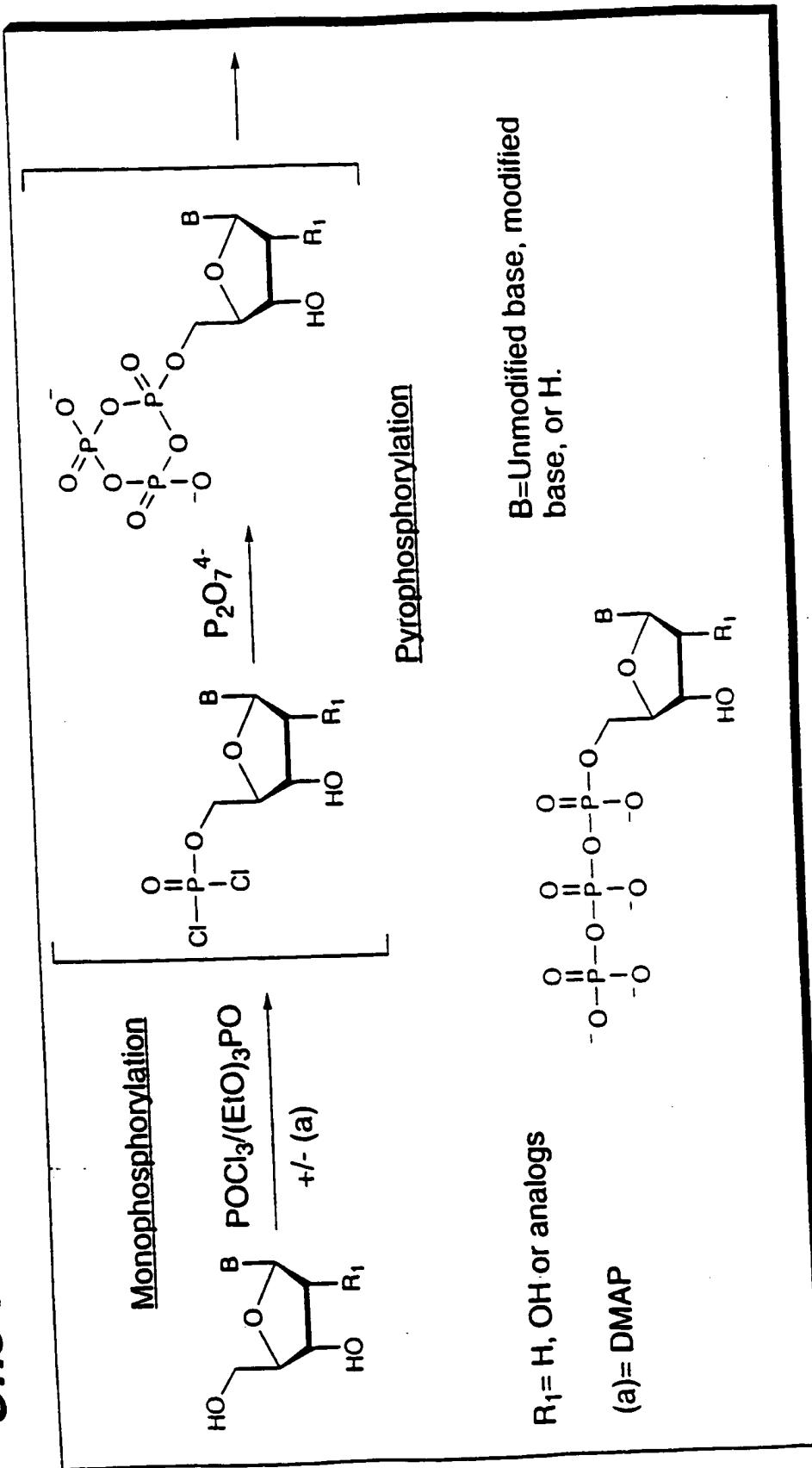


Figure 28. RPA Demonstrating Inhibition of Cellular Bcl-2 mRNA Using Bcl-2 Directed Ribozymes.





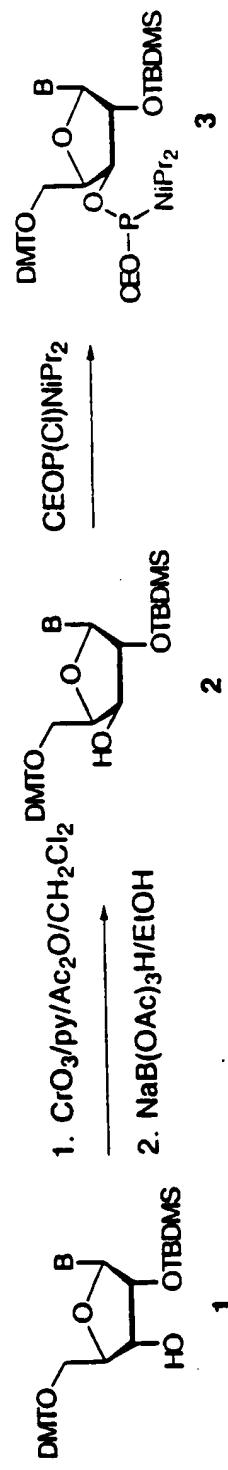
**Fig. 29**  
**One-Pot Formation of Nucleosides-5'-Triphosphates**





**Figure 30: Synthesis of a Xylo ribonucleoside phosphoramidite**

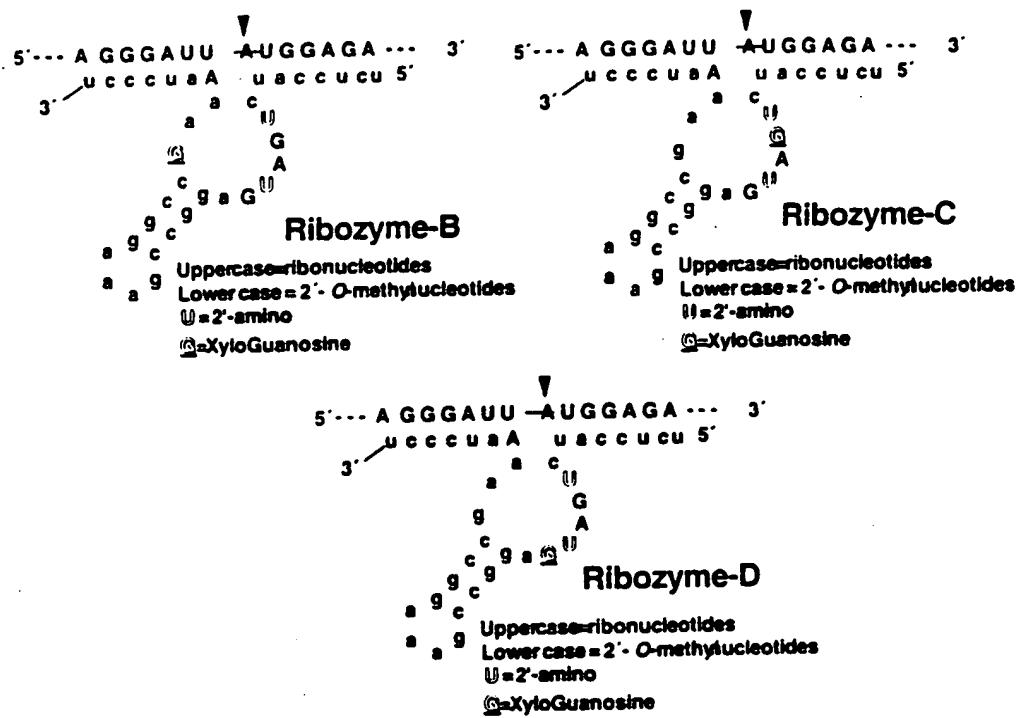
**Scheme 1**



**B** = standard or modified nucleotide base or H

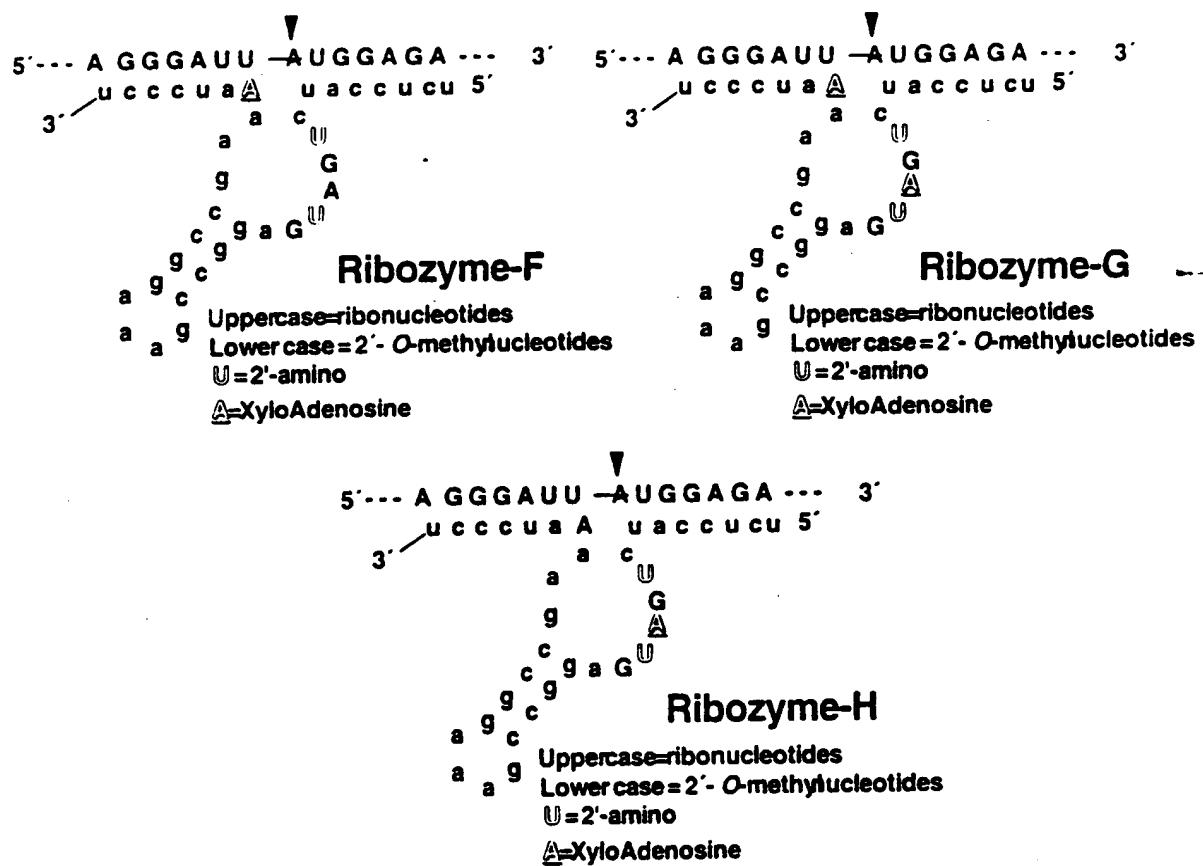


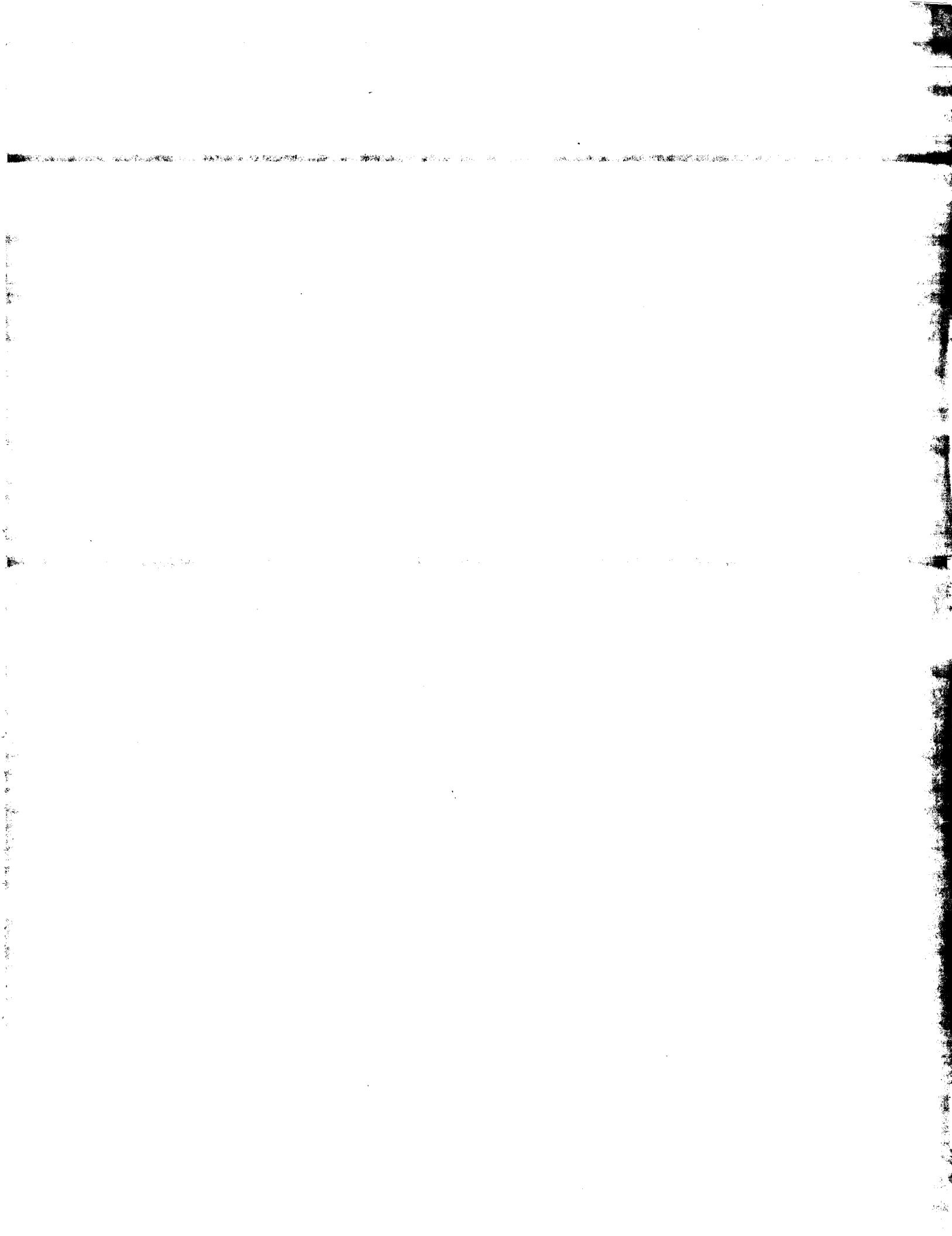
**Figure 31A: Xylo Nucleotide-Substituted Hammerhead Ribozymes**



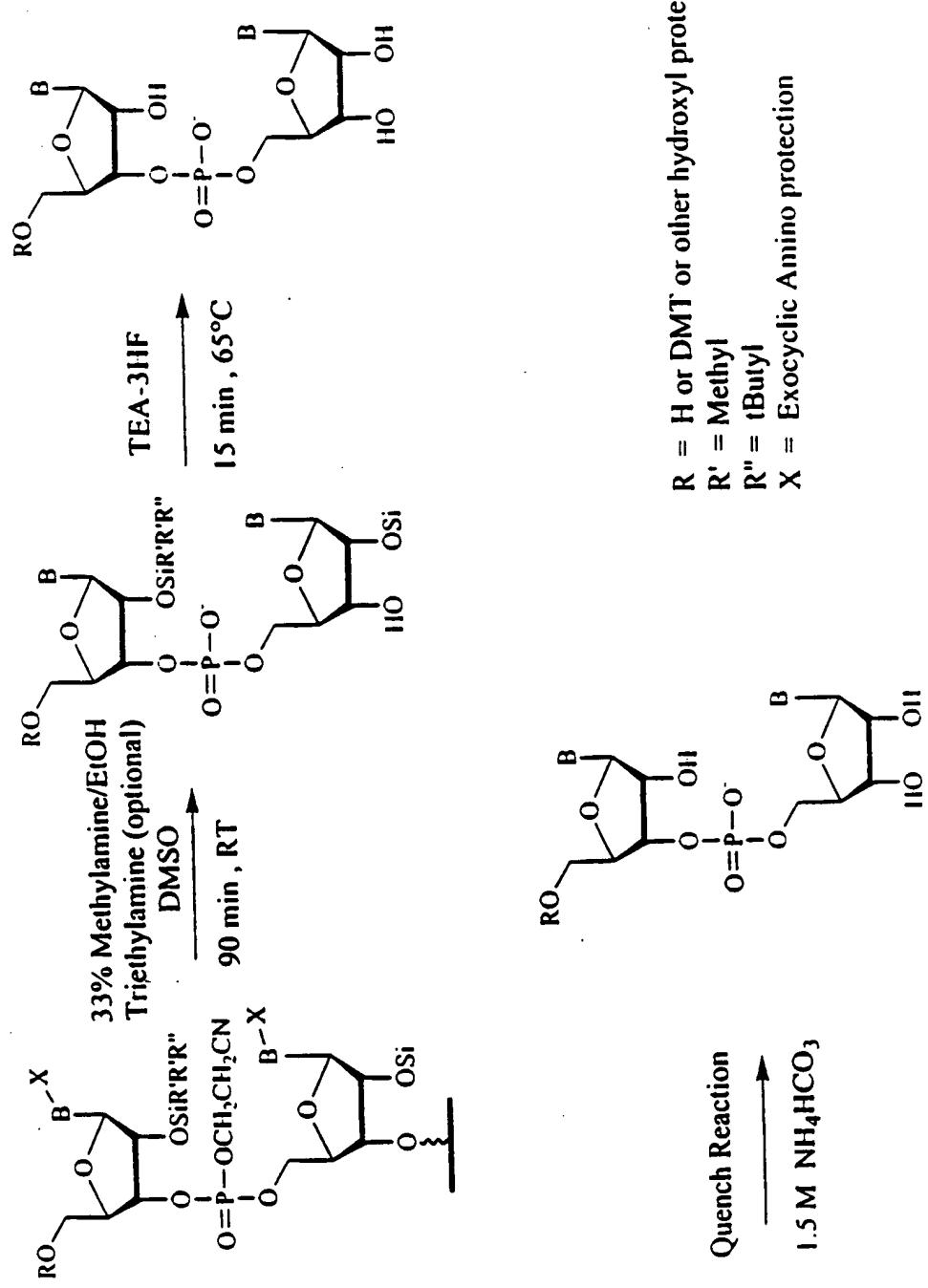


### Figure 31B: Xylo Nucleotide-Substituted Hammerhead Ribozymes





**Figure 32: Improved One Pot Deprotection of RNA**





Overlay of chromatograms of  
or "two-pot" deprotected RPI.3703 ribozyme

RPI.3703 guuu<sub>s</sub> uucc<sub>u</sub> GAu Gg gc<sub>c</sub> ag gc<sub>g</sub> aa<sub>A</sub> uuc uc<sub>B</sub> 200 n mole scale

**One-pot      Two-pot**

**Base Deprotection:**

400  $\mu$ L MA/EtOH/TEA 1 mL MA/H<sub>2</sub>O  
10 min @ 65 °C

400  $\mu$ L DMSO  
90 min @ RT  
Dry down

**Silyl Deprotection:**

300  $\mu$ L TEA:3HF/NMP  
90 min @ 65 °C

**Quench:**  
1mL 1.5M NH<sub>4</sub>HCO<sub>3</sub>  
1mL 1.5M NH<sub>4</sub>HCO<sub>3</sub>

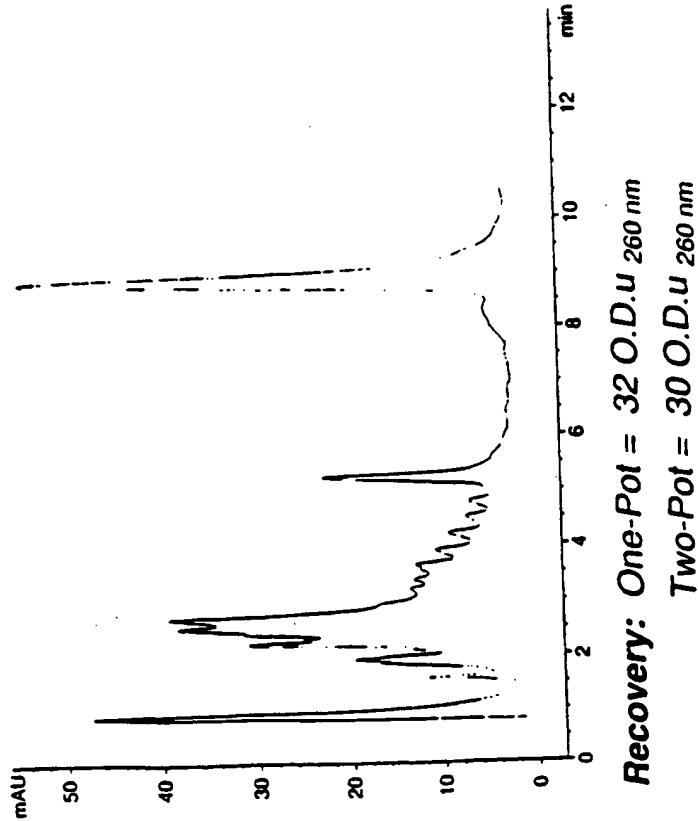
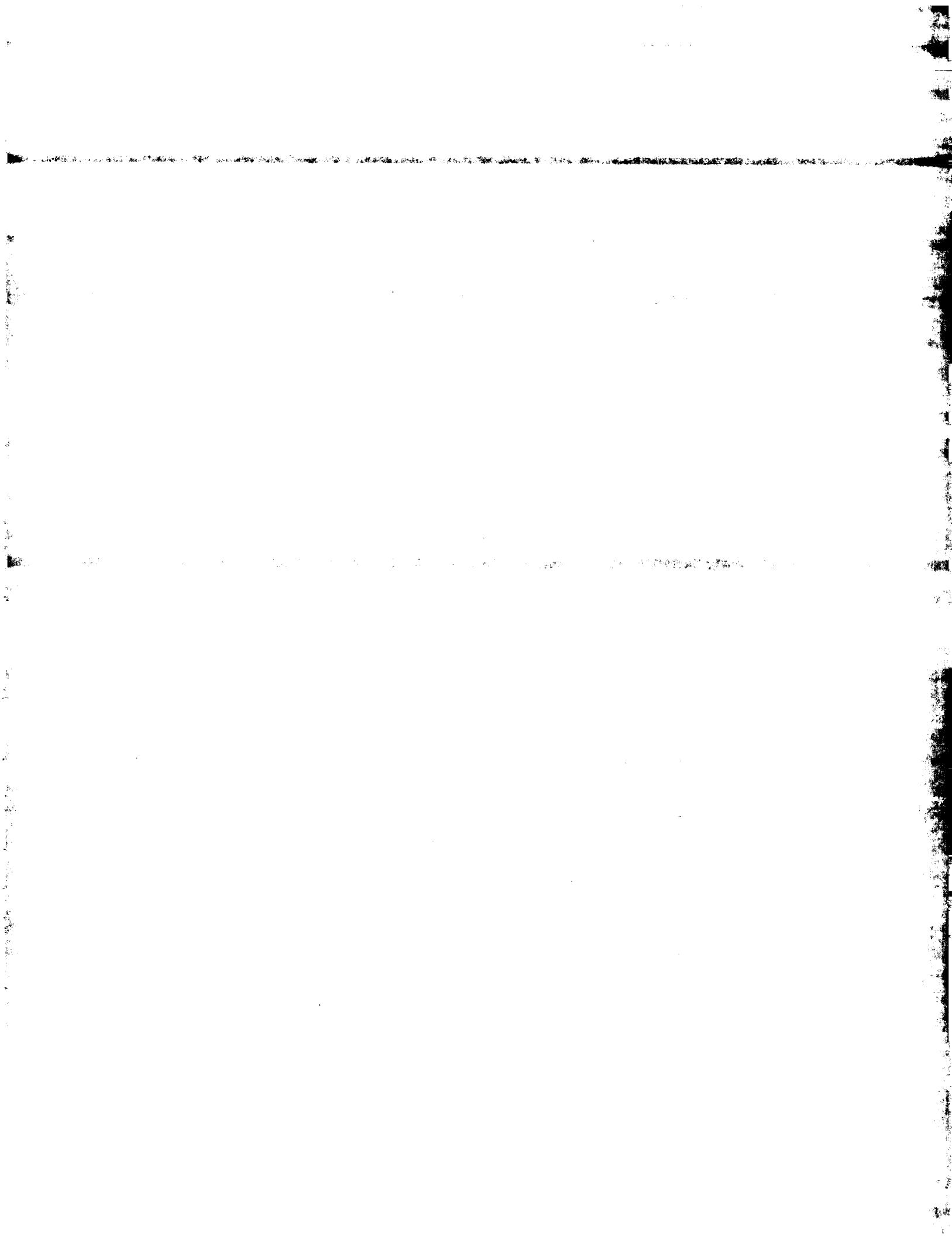
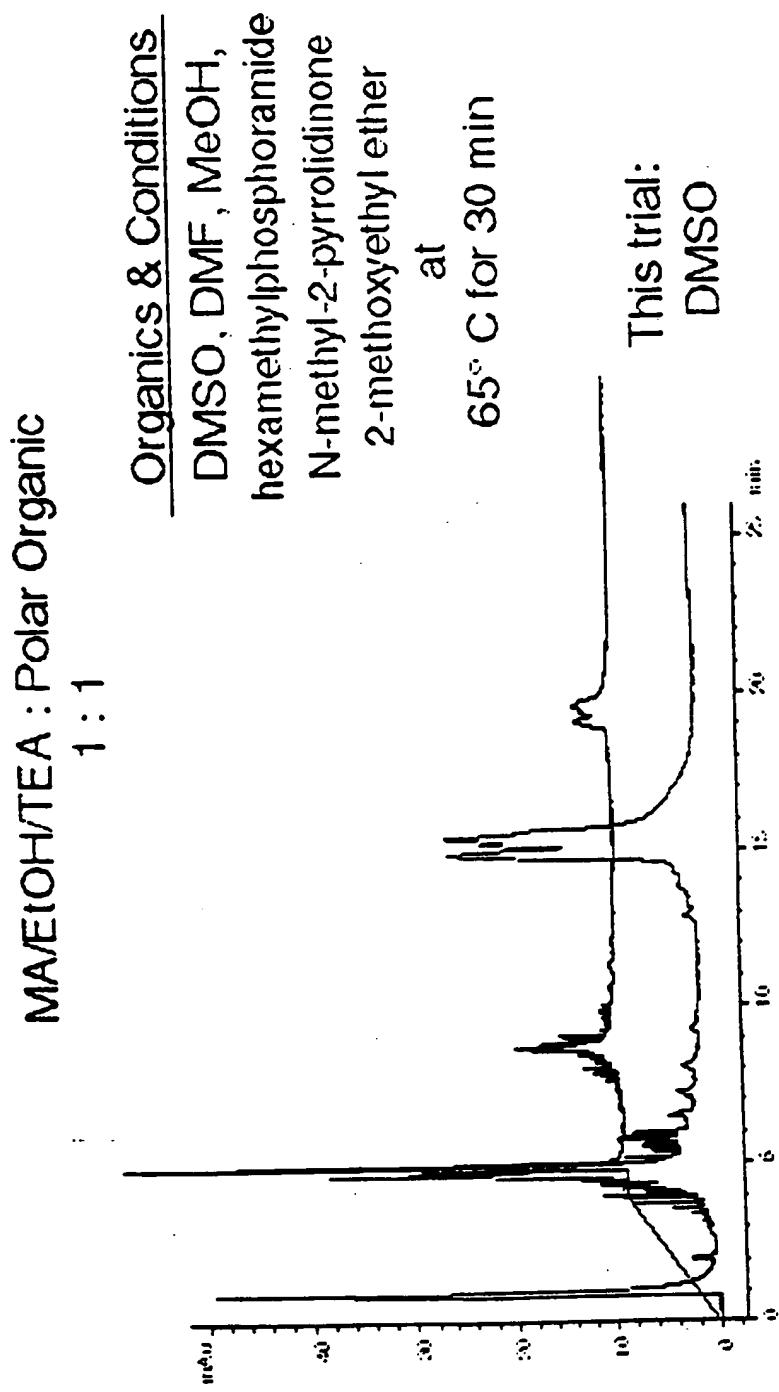


Figure 33



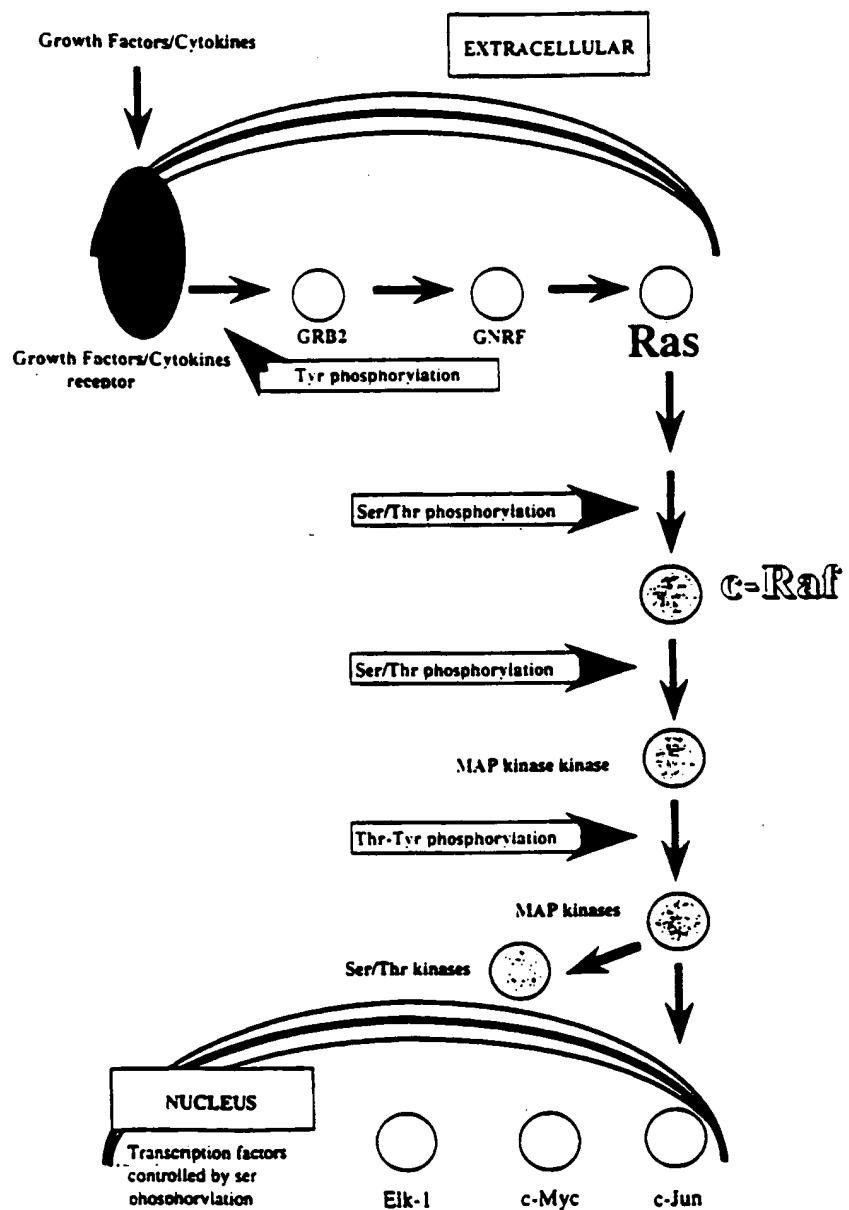


Only DMSO permitted the release of oligo.

Figure 34



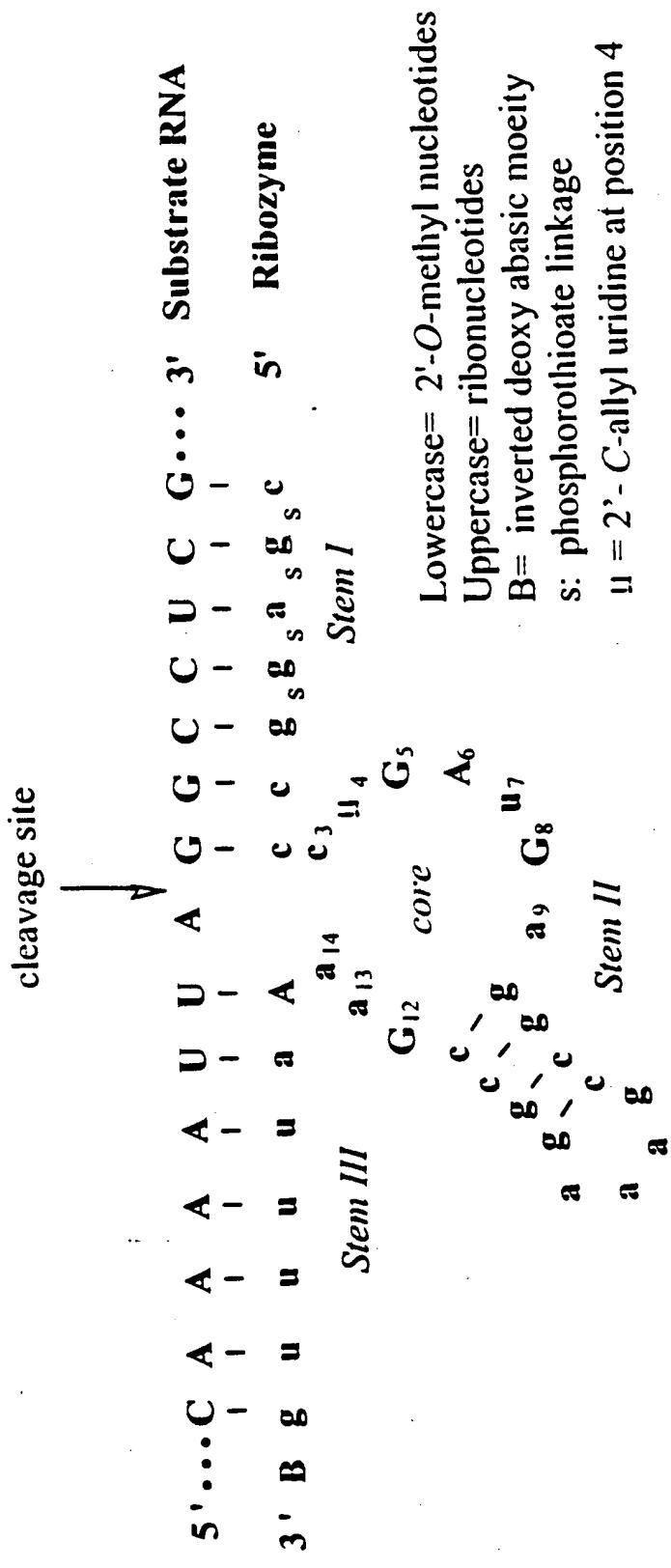
**Figure 35: Ras-Dependent Signal Transduction cascade**



Adapted from *Genes V*, Lewin, 1994 Oxford University Press, New York, pp 1211



Figure 36: Structure of ribozyme directed to site 1120 and its substrate RNA sequence



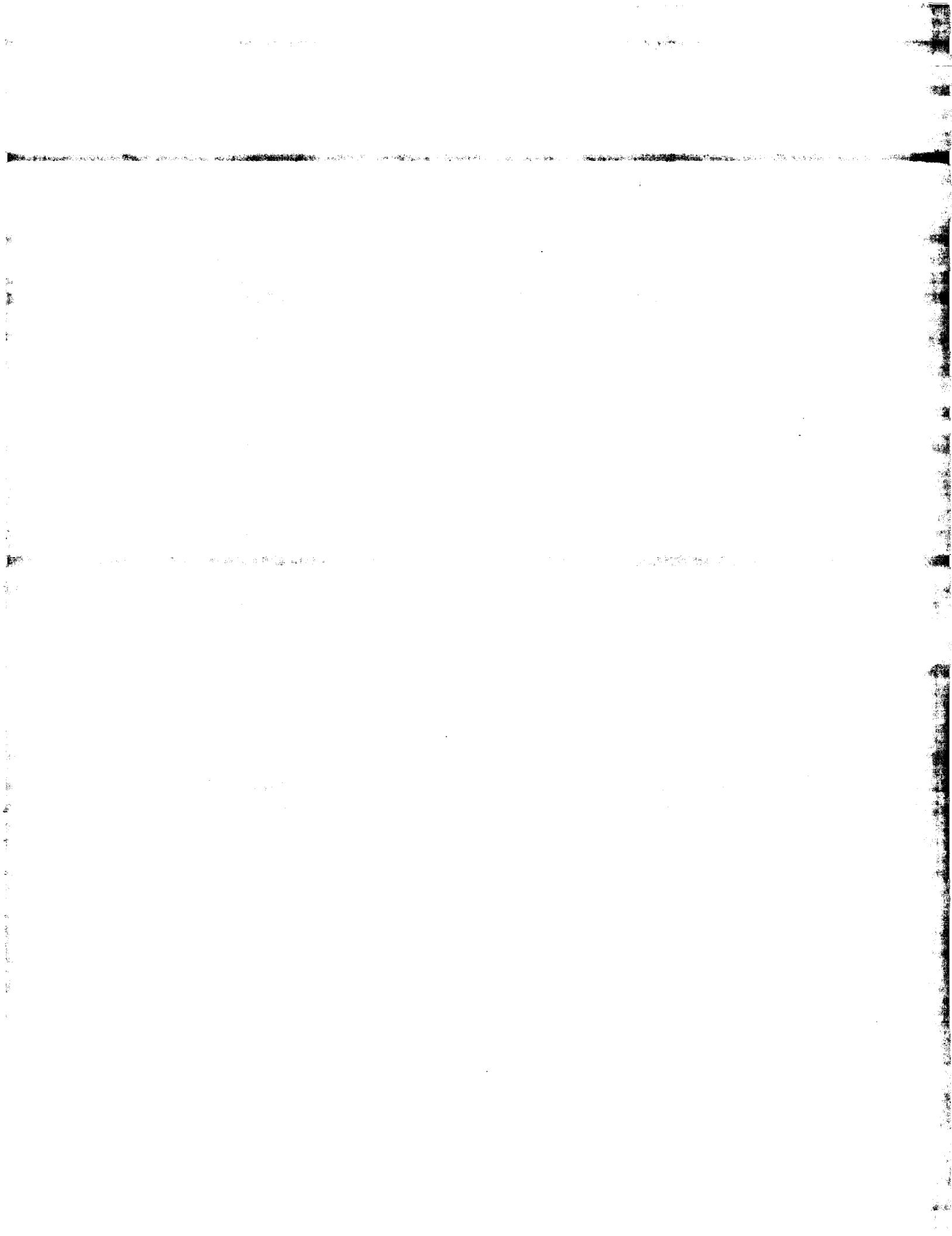


Figure 37: c-raf Ribozyme-Mediated Inhibition of Smooth Cell Proliferation

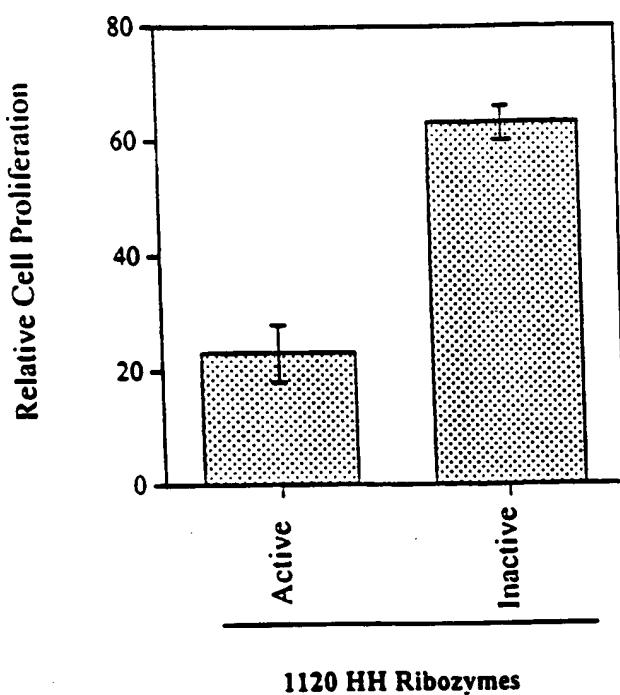
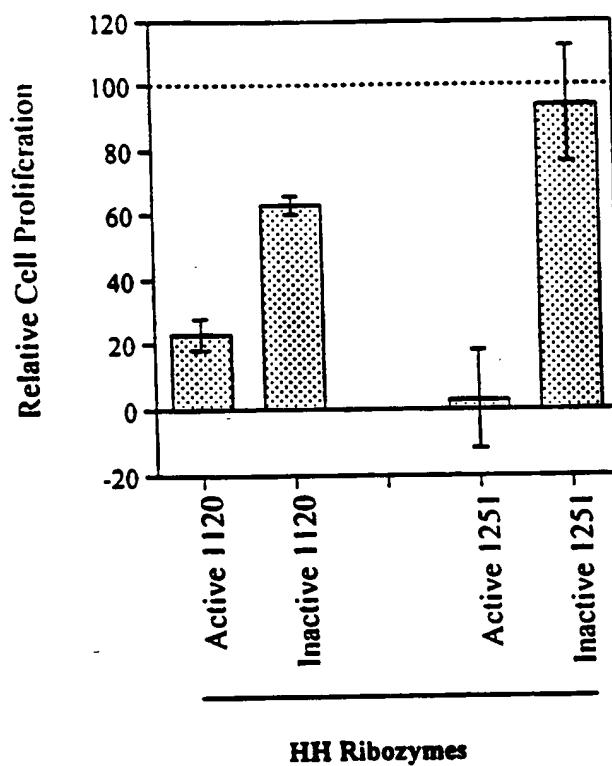
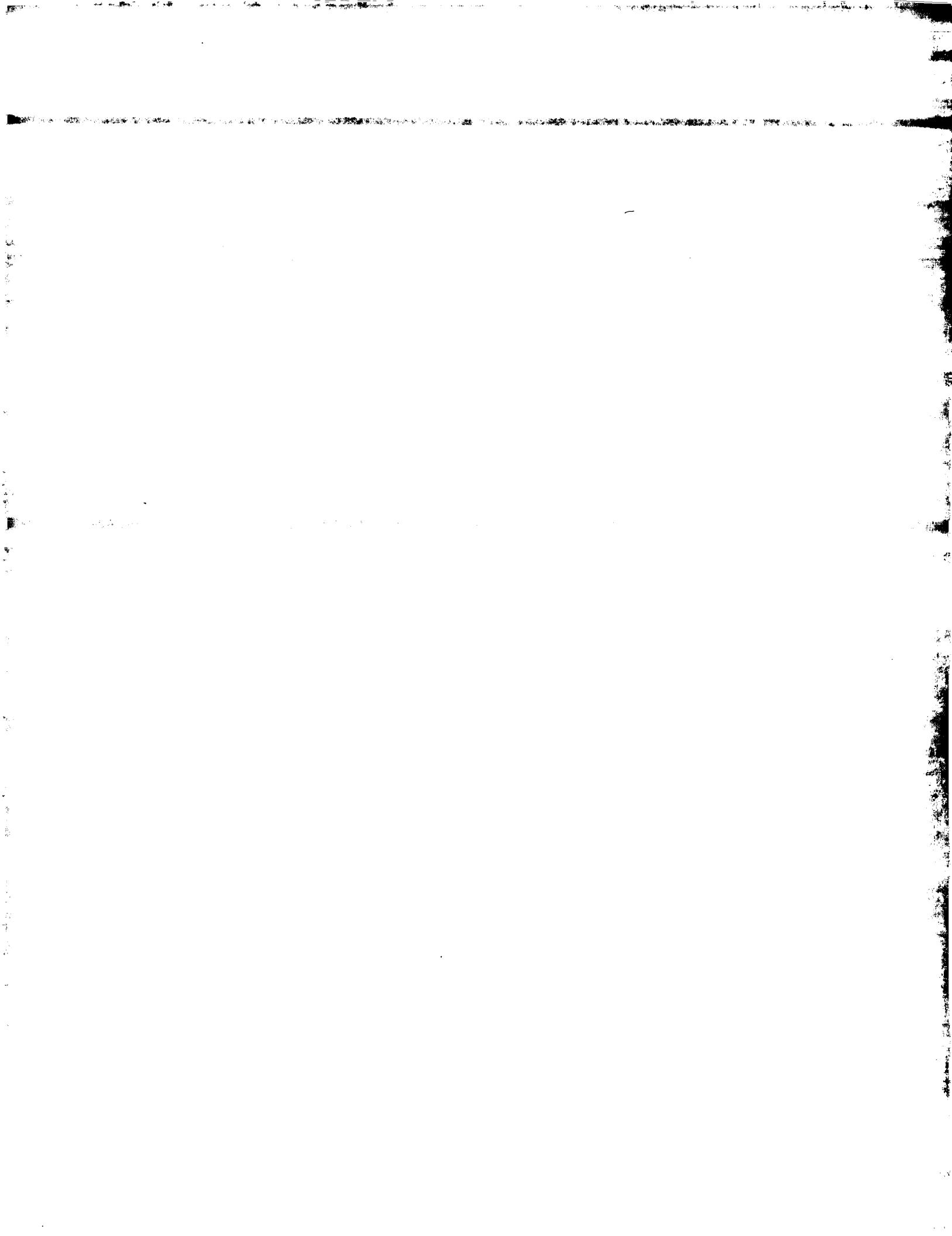


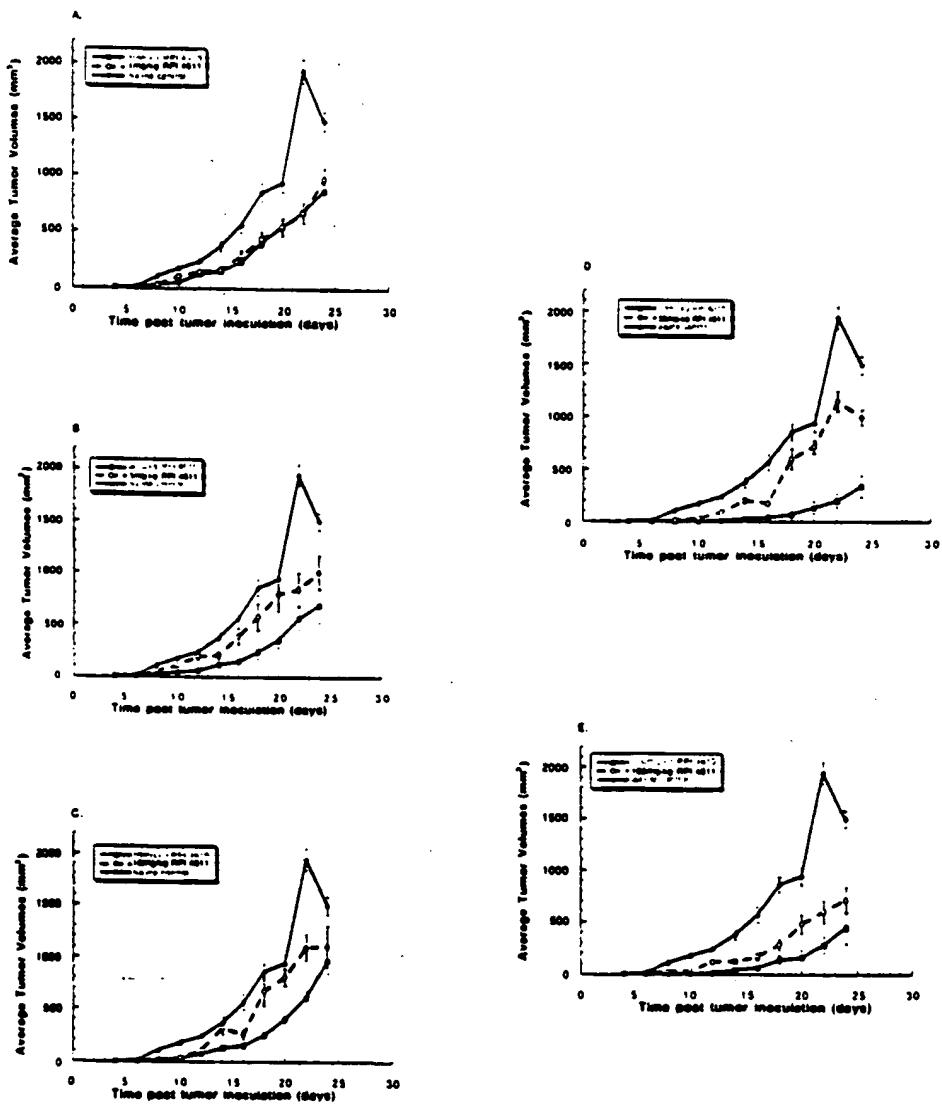


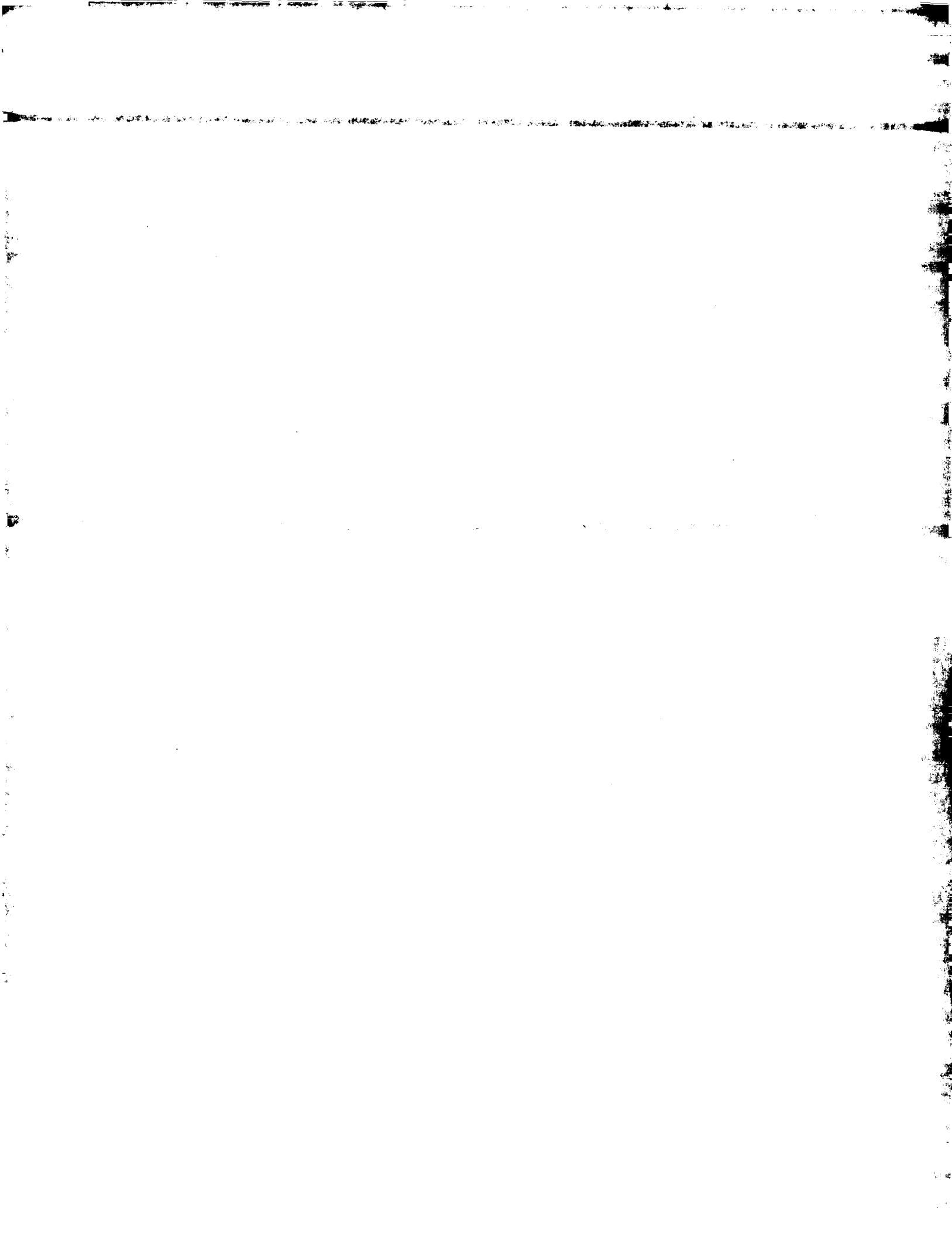
Figure 38: c-raf Ribozyme-Mediated Inhibition of Smooth Cell Proliferation



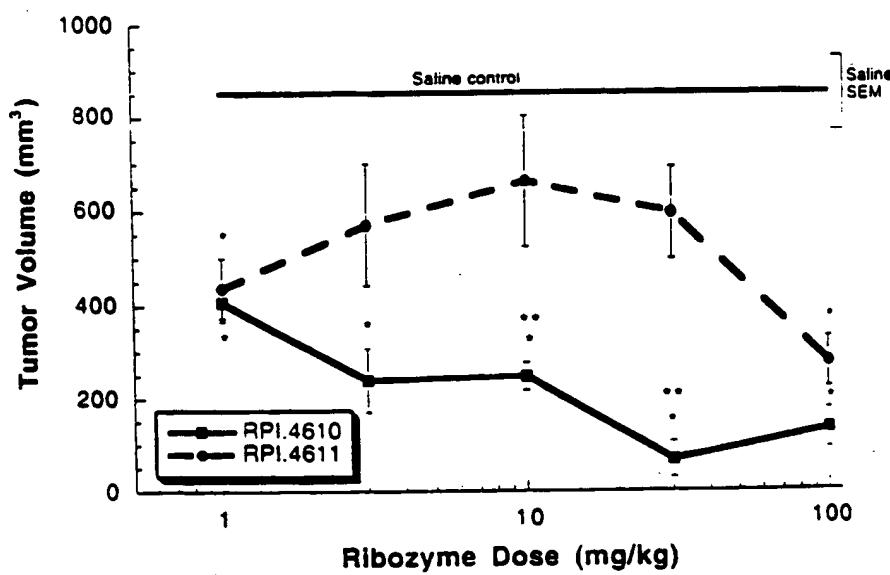


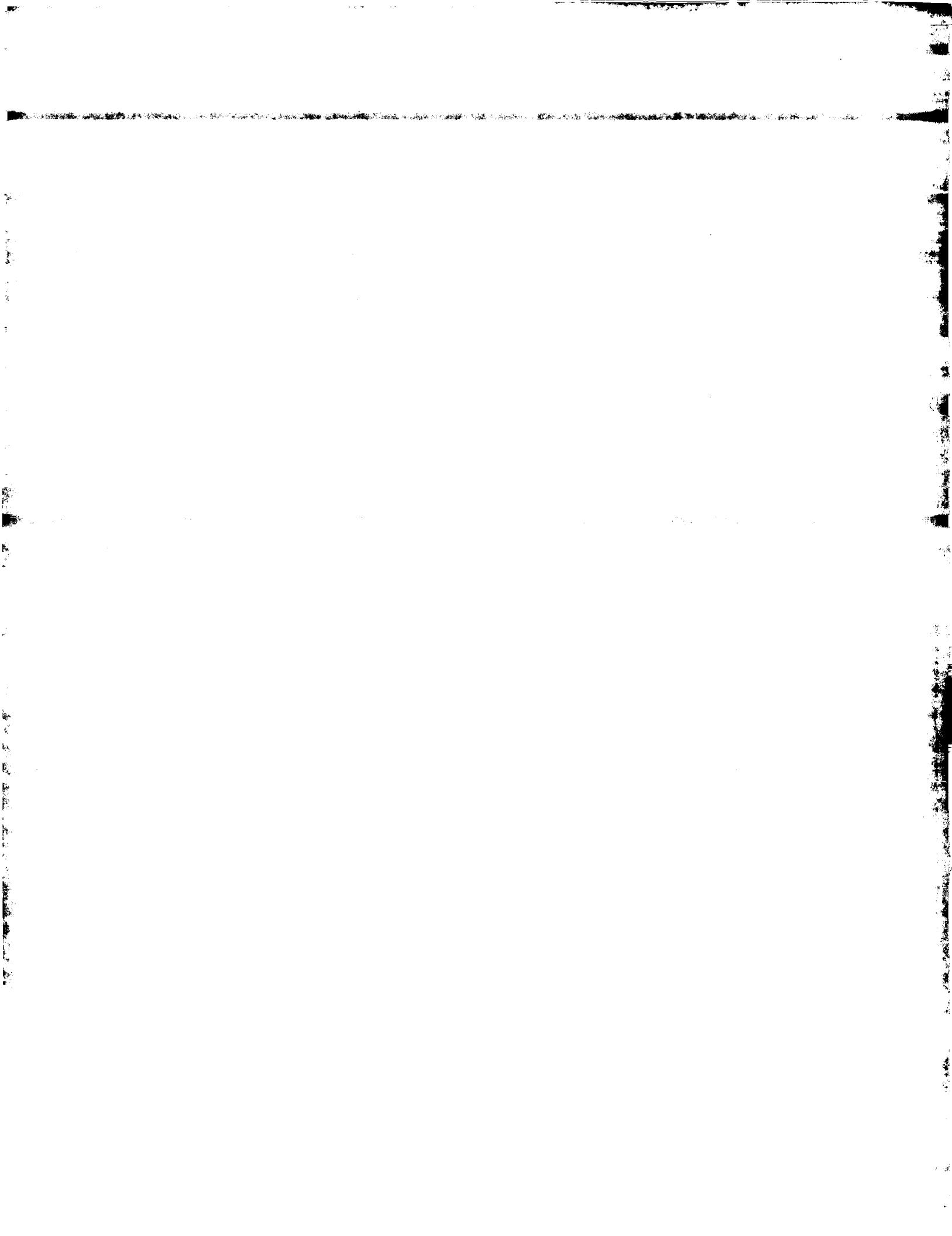
**Figure 39. Effects of *flt-1* ribozymes (active/inactive) on LLC-HM primary tumor growth in mice.** A. 1, B. 3, C. 10, D. 30, E. 100 mg/kg/day *flt-1* ribozyme dose. Treatment begins on day 3 and continues through day 17. Data are reported as mean tumor volume  $\pm$  SEM.



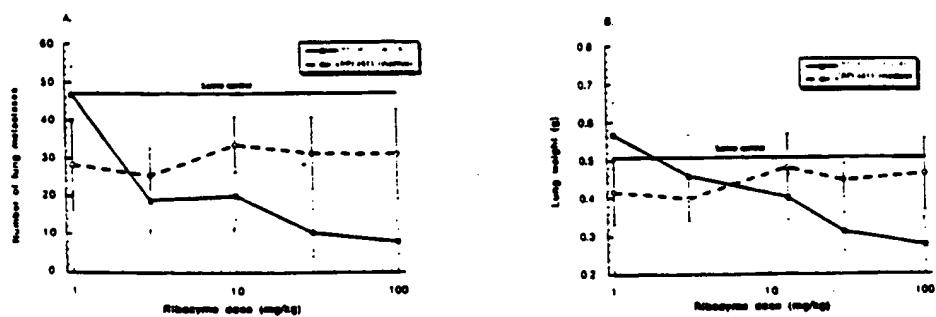


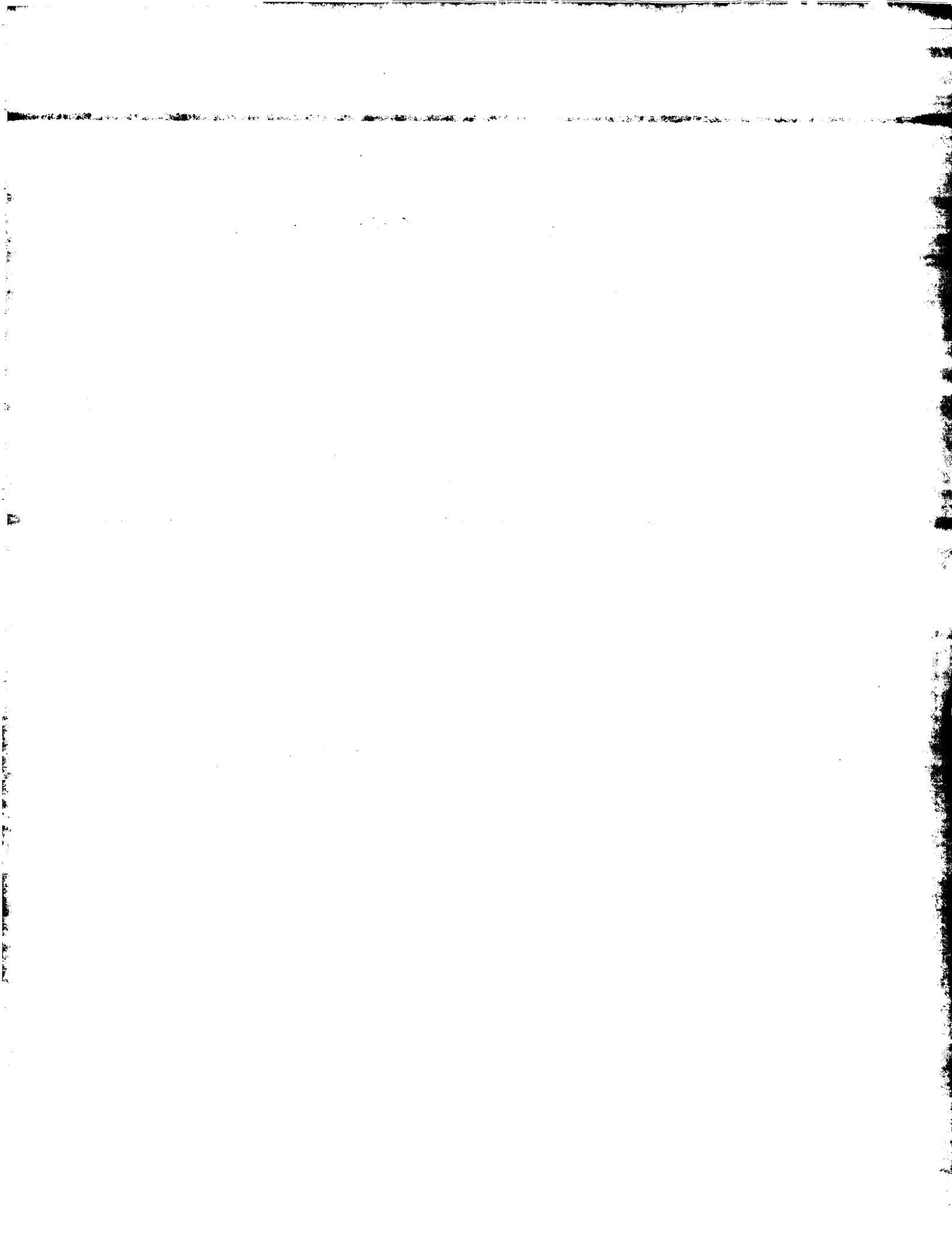
**Figure 40. Effects of *flt-1* ribozymes on LLC-HM primary tumor volume immediately following the cessation of treatment. RPI.4610 is catalytically active/RPI.4611 is catalytically inactive. Shaded region denotes saline control SEM. Data are reported as mean tumor volume  $\pm$  SEM. \*  $p < 0.05$  with respect to control by Dunnets. \*\*  $p < 0.05$  with respect to inactive by Tukey-Kramer.**



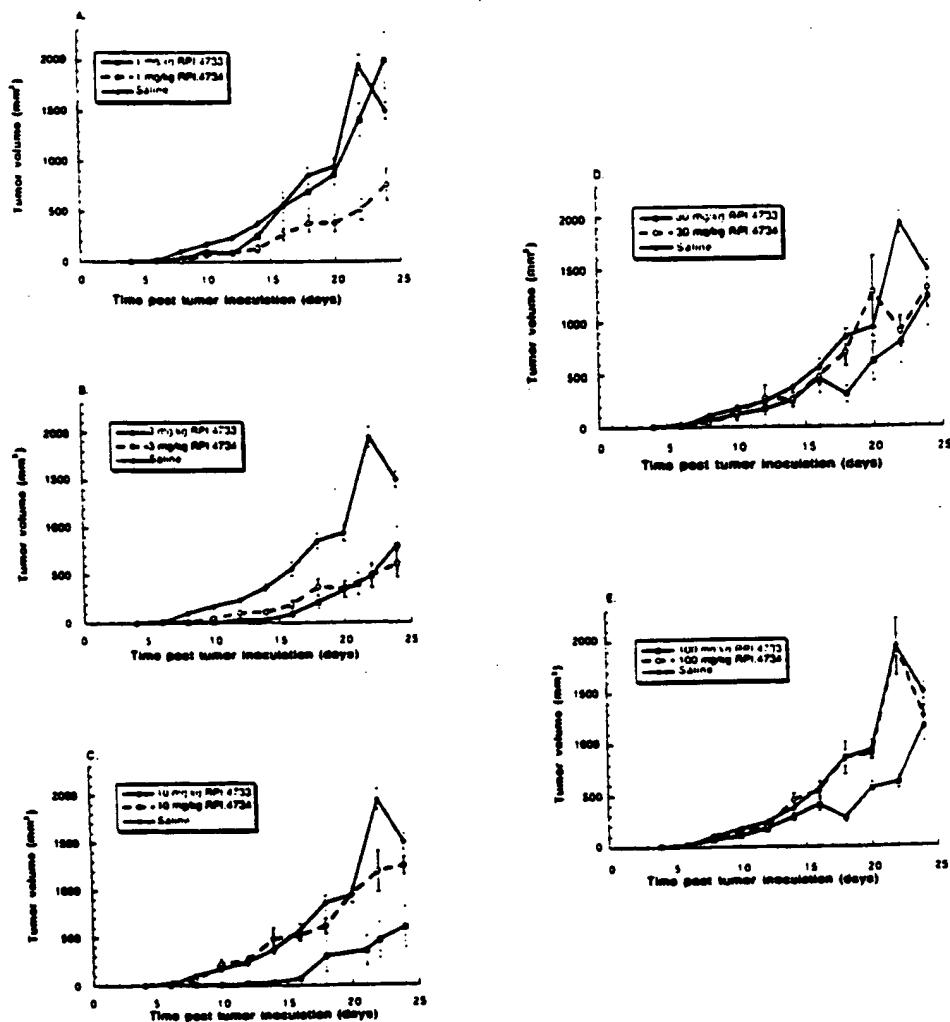


**Figure 41. Effects of *flt-1* ribozymes on lung metastatic indices (number of metastases and lung mass). A. Number of lung metastases. B. Lung mass. Data are presented as mean number of metastases or lung mass  $\pm$  SEM. Shaded region denotes saline control SEM. \* p < 0.05 with respect to saline control by Dunnett's. † p < 0.05 with respect to saline control by Student's t.**



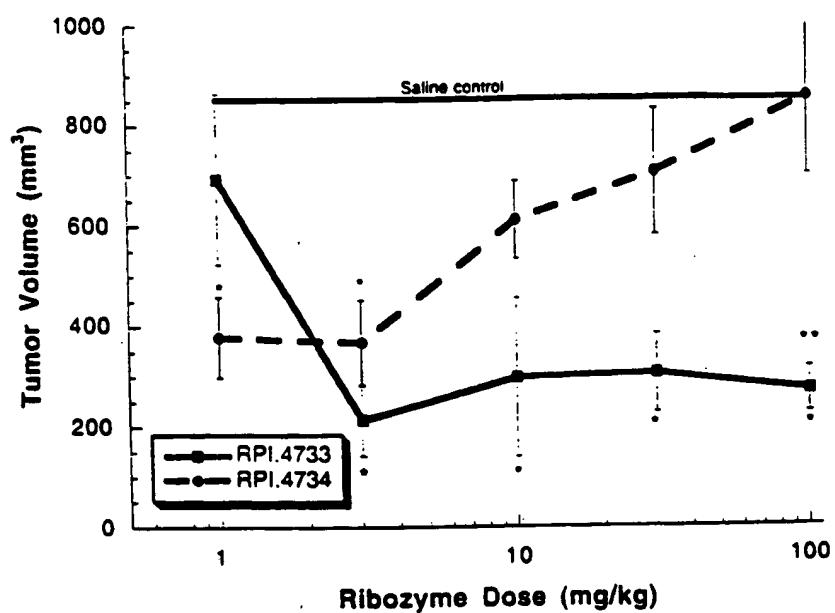


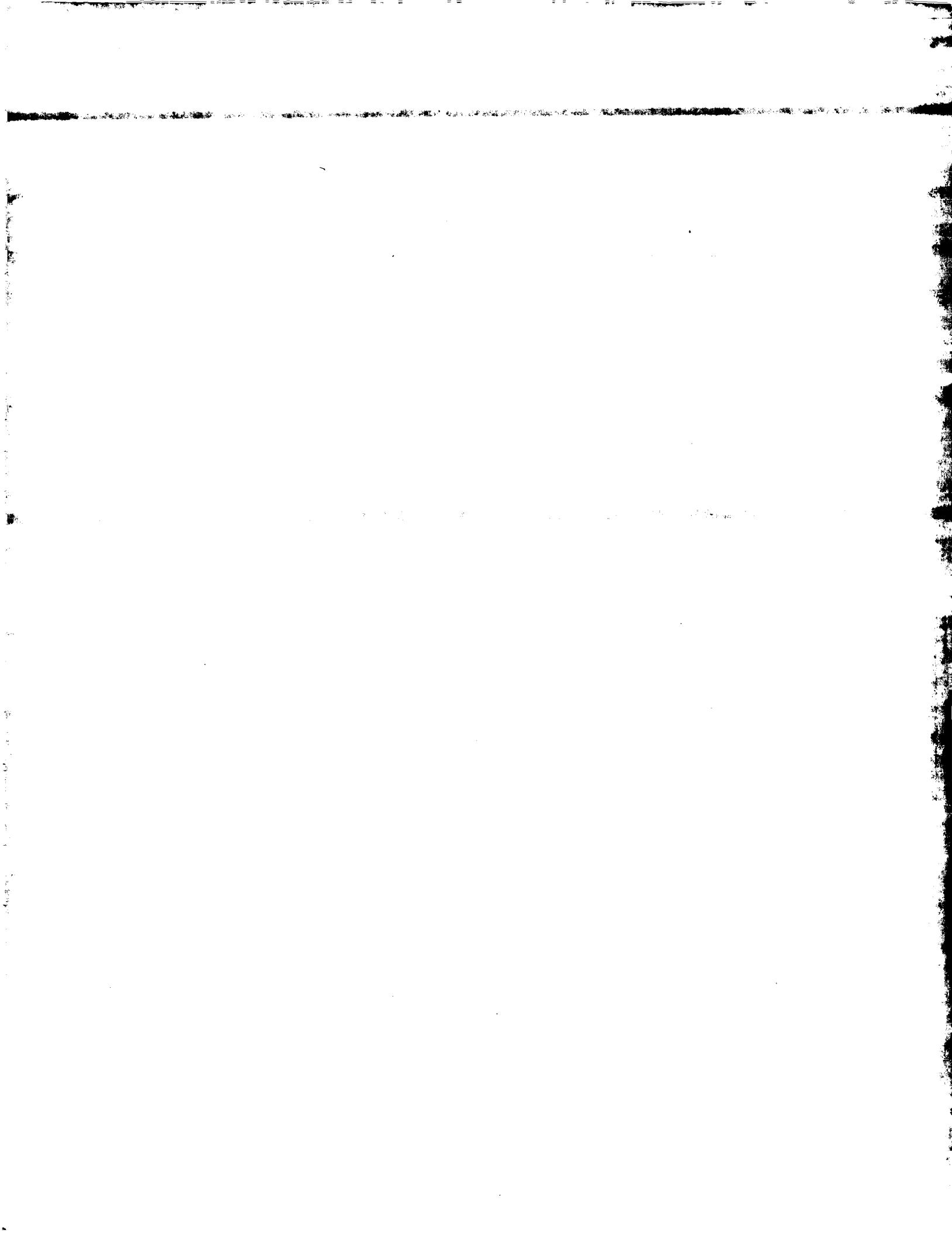
**Figure 42. Effects of *flk-1* ribozymes (active/inactive) on LLC-HM primary tumor growth in mice. A. 1, B. 3, C. 10, D. 30, E. 100 mg/kg/day *flk-1* ribozyme dose. Treatment begins on day 3 and continues through day 17. Data are reported as mean tumor volume  $\pm$  SEM.**





**Figure 43. Effects of *flk-1* ribozymes on LLC-HM primary tumor volume immediately following the cessation of treatment. RPI.4733 is catalytically active/RPI.4634 is catalytically inactive. Shaded region denotes saline control SEM. Data are reported as mean tumor volume  $\pm$  SEM. \*  $p < 0.05$  with respect to control by Dunnett's. \*\*  $p < 0.05$  with respect to inactive by Tukey-Kramer.**





**Figure 44. Effects of *flk-1* ribozymes on lung metastatic indices (number of metastases and lung mass). A. Number of lung metastases. B. Lung mass. Data are presented as mean number of metastases or lung mass  $\pm$  SEM. Shaded region denotes saline control SEM. \*  $p < 0.05$  with respect to saline control by Dunnett's, †  $p < 0.05$  with respect to saline control by Student's t, \*\*  $p < 0.05$  with respect to inactive control.**

